INTRON-MEDIATED ENHANCEMENT OF THE EXPRESSION OF HETEROLOGOUS PROTEIN IN PLANT

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UNDER THE GUIDANCE

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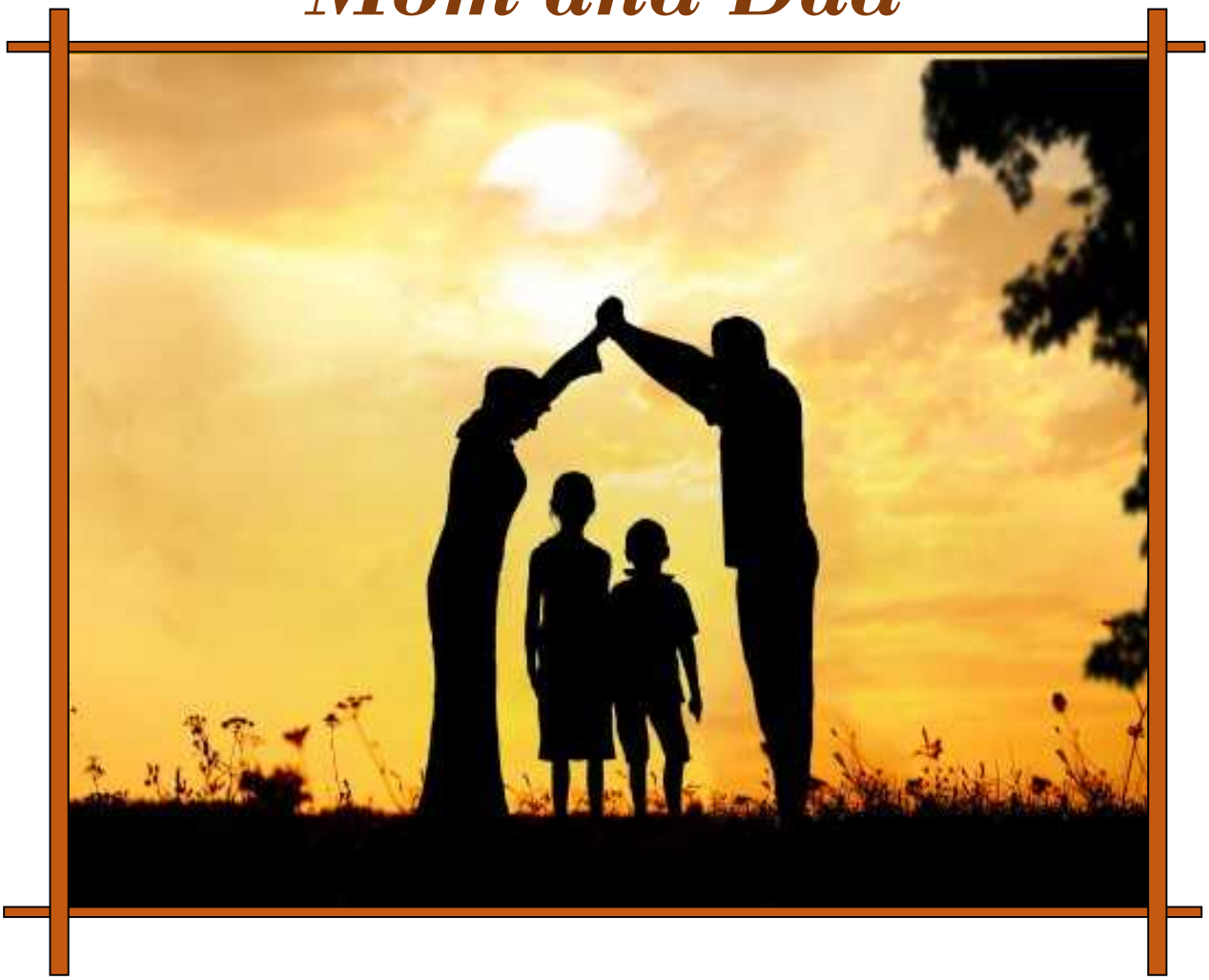
One of the greatest titles in the world is parent, and one of the biggest blessings in the world is to have parents to call

"Mom and Dad"



Dedicated to Beloved Parents

&

Adorable Brother…..!!!

"A tribute to my **Mother"**

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A. tumefaciens	:	*Agrobacterium tumefaciens*
ABRC	:	Arabidopsis Biological Resource Centre
ALS	:	Alkaline lysis solution
APS	:	Ammonium persulfate
BA	:	6- benzyladenine
Bar	:	Bialaphos resistance
bp	:	Base pairs
BPB	:	Bromophenol Blue
$CaCl_2.2H_2O$	:	Calcium chloride 2-hydrate
CaMV	:	Cauliflower mosaic virus
cDNA	:	Complimentary DNA
Cf	:	Cefotaxime
$CoCl_2.6H_2O$	:	Cobalt chloride 6-hydrate
$CuSO_4.5H_2O$	:	Copper sulphate 5-hydrate
Da	:	Dalton
DDW	:	Double distilled water
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxy nucleotides tri-phosphate
E. coli	:	*Escherichia coli*
EDTA	:	Ethylenediaminetetraacetic acid
EPO	:	Erythropoietin
ER	:	Endoplasmic reticulum
FDA	:	Food Drug Administration
$FeSO_4.7H_2O$	:	Ferrous sulphate 7-hydrate
g	:	Gravitational force
gm	:	Gram
GP	:	Glycoprotein
H_3BO_3	:	Boric acid
HCl	:	Hydrochloric acid

HPLC	:	High performance liquid chromatography
hr	:	Hours
IAA	:	Indole 3-acetic acid
IPA	:	Isopropyl alcohol
Kb	:	Kilo basepair
kDa	:	Kilo Dalton
KI	:	Potassium iodide
Kn	:	Kanamycin
KNO_3	:	Potassium nitrate
LA	:	Luria agar
LB	:	Luria broth
M	:	Molar
MAS	:	Mannopine synthase
mg/L	:	Milligram per litre
mg/ml	:	Milligram per millilitre
$MgSO_4.7H_2O$	:	Magnesium sulphate 7-hydrate
min	:	Minute
ml	:	Millilitre
mM	:	Millimolar
$MnSO_4.4H2O$	:	Manganese sulphate 4-hydrate
MS medium	:	Murashige and Skoog's medium
N. tabacum	:	*Nicotiana tabacum*
$Na_2EDTA.2H_2O$	:	Di-sodium EDTA
$Na_2MoO_4.2H_2O$	:	Di-sodium molybdate 2-hydrate
NAA	:	1-Naphthaleneacetic acid
NaOH	:	Sodium hydroxide List of Abbreviations
ng	:	Nanogram
NH_4NO_3	:	Ammonium nitrate
nm	:	Nanometre
NptII	:	Neomycin phosphotransferase
OD	:	Optical density
p	:	Plasmid

PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PGRs	:	Plant growth regulators
pH	:	Hydrogen ion concentration
psi	:	Pounds per square inch
rDNA	:	Recombinant DNA
RE	:	Restriction Endonuclease
rpm	:	Revolutions per minute
Rt	:	Retention time
S. cerevisiae	:	*Saccharomyces cerevisiae*
SDS	:	Sodium dodecyl sulfate
Sec	:	Second
STE	:	Sodium-Tris-EDTA
Syn	:	Synthetic
TAE	:	Tris-acetate EDTA
T-DNA	:	Transferred DNA
TE	:	Tris EDTA
UV	:	Ultraviolet
V	:	Volt
$ZnSO_4.7H_2O$	:	Zinc sulphate 7-hydrate
µF	:	Micro farad (unit of capacitance)
µg/ml	:	Microgram per millilitre
µg/µl	:	Microgram per microlitre
µM	:	Micromolar
Ω	:	Ohm

Intron-mediated enhancement of the expression of heterologous protein in plant

Abstract

Advances in the biotechnological approaches have led to exploration of various techniques and strategies for overexpression of heterologous proteins in plant system. One such strategy is intron-mediated enhancement (IME), which is being used for enhanced expression of recombinant proteins. Introns are the non-functional part of genomic DNA that is unable to code any protein. However, its presence in genomic DNA has attracted researchers to explore the mysterious nature of intron. Earlier studies in mammalian cells have revealed the regulatory role of intron in expression of gene. Further, the regulatory effect of intron on gene expression was also explored in plants. Subsequently, many studies were performed in plants for enhancement of transgene expression. In the present study, IME strategy was used to enhance the expression of erythropoietin (EPO) in *Nicotiana tabacum* plant using Synthetic 7 (Syn7) intron. *Agrobacterium*-mediated genetic transformation was used for transformation of *N. tabacum*. For the comparative analysis of EPO expression, two vector constructs namely control expression vector (without intron) and test expression vector (with intron) were prepared. Integration of EPO in genomic DNA of transgenic plants was analysed using PCR and southern blot analysis. Indirect confirmation of transgenic *N. tabacum* was done by studying the metabolic burden in transgenic plants using HPLC analysis of major alkaloid, Nicotine. Expression of EPO at transcriptional and translational level was analysed using Real time PCR and ELISA assay, respectively. The expression of EPO was increased 2-17 fold at mRNA level and 9 fold at protein level in the presence of intron Syn7 as compared to the intron less construct. Based on the results, it can be concluded that intron-mediated enhancement strategy was found efficient to enhance the expression of EPO in *N. tabacum*.

Chapter 1

Introduction

1.1 Heterologous proteins

The advent of rDNA technology has facilitated the expression of a gene of interest in a non-native living system (the host system) for human benefits. A protein produced by a non native gene in a host system is known as heterologous proteins. There are many non-native host systems available and used for production of various heterologous proteins at commercial scale.

1.2 Host systems

Production of heterologous proteins involves various steps which starts with the procurement of gene from the source to its cloning in appropriate vector and finally based on the complexity (post translational modification) of the protein, it ends up with the expression in suitable host systems **(Figure 1.1).** Glycosylation is one of the most important post translational modification and also a deciding factor in the selection of host system for expression of heterologous proteins. Non-glycosylated proteins are generally expressed in the prokaryotic expression systems while glycosylated proteins are expressed in eukaryotic systems. A brief account of various types of host systems is given below-

1.2.1 Bacteria

In prokaryotes, *Escherichia coli* bacteria are one of the most common and widely used host systems. Rapid growth, ease of handling, easier genetic manipulation and well studied genome make *E. coli* a good host system for heterologous protein expression (Swartz 1996; Terpe 2006). There are many strains of *E. coli* which are being used as expression host such as *E. coli DH5α, E .coli BL21* and *E. coli K12*. A number of proteins such as insulin, human growth hormone, α, β, γ-interferons and G-CSF have been expressed successfully in *E. coli* (Swartz 1996). In case of *E. coli* bacterial cells, it has been observed that proteins are

expressed in the form of inclusion bodies which increases the cost of downstream processing (Fischer et al. 1993).

Many of *Bacillus* strain e.g. *B. subtilis, B. brevis,* and *B. licheniformis* are also found compatible for the heterologous proteins production (Terpe 2006). They can secrete heterologous proteins directly into medium which makes subsequent downstream processing effortless and cost effective. However, presence of various proteases in cells sometimes degrades heterologous proteins or provides low production.

One of the major limitations associated with bacterial host system is that, it cannot produce glycosylated protein with proper folding which leads to production of biologically inactive proteins.

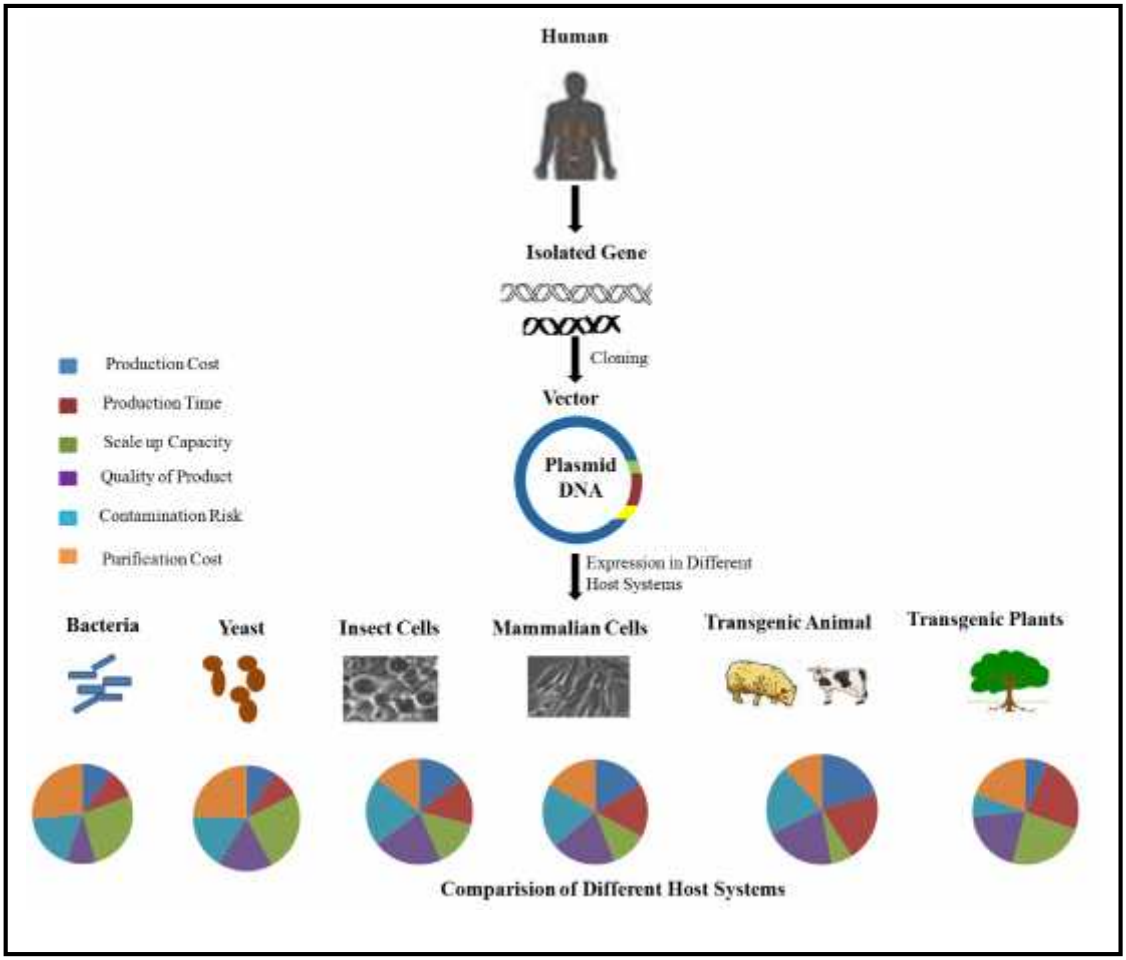


Figure 1.1: Process of gene cloning, expression and comparison of different host systems in terms of protein production

1.2.2 Yeast

Limitations of proper folding and glycosylation of proteins in bacterial cells (prokaryotic system) encourage the expression of glycosylated proteins in yeast cells (eukaryotic system). Yeasts are single celled eukaryotes which has capacity of glycosylation. *Saccharomyces cerevisiae* and *Pichia pastoris* are the commonly used yeasts for heterologous protein expression. Sequenced genome information is an added advantage for genetic manipulation of the organisms. Nearly, similar (human system) posttranslational modifications, rapid and higher growth rate and cost effectivity are some advantageous features, which make them more preferable host system than the bacterial cells for the production of glycosylated heterologous proteins. Insulin, hepatitis B surface antigen, urate oxidase, glucagons, granulocyte macrophage colony stimulating factor (GM-CSF), hirudin, and platelet-derived growth factor are the examples of heterologous proteins which have been produced in yeasts cells. However, this system also suffer with some limitations like presence of only mannose sugar, absence of sialyted o-linked chain, occurrence of over-glycosylated N-linked sites which are responsible for reduced activity of the proteins which lead to immunological problems (Kukuruzinska et al. 1987; Kornfeld and Kornfeld 1985). Furthermore, yeast as a host system also has limitations of low product yield and inefficient secretion of protein (Dominguez et al. 1998).

1.2.3 Insect

Insect cells as host system for heterologous protein are able to perform complex post translational modifications (Agathos 1991). Protein expression in insect cells is generally carried out using baculovirus as a vector, which does not harm mammalian cells. It facilitates proper protein folding and the higher expression of proteins with posttranslational, modifications such as N- and O-linked glycosylation, correct signal peptide cleavage, proper proteolytic processing, acylation, phosphorylation, carboxymethylation, and prenylation (Luckow and Summers 1988; Miller 1988). However, limitation of this system is, it cannot process complex type oligosaccharides containing fucose, galactose and sialic acid (Rai and Padh 2001).

1.2.4 Mammalian cells

Mammalian cells are the most popular host system for heterologous protein expression specially those of therapeutic importance. This system provides high level of protein expression, proper protein folding with the mammalian post-translational modifications which are required for protein to be biological active. Most of the commercially available proteins are being expressed in mammalian cells such as glycosylated fertility hormones, erythropoietin, human chorionic gonadotropin, human luteinizing hormone etc. Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, green monkey kidney cells and human cell lines e.g. human embryonic kidney (HEK) cells are used for the expression of heterologous proteins (Demain and Vaishnav 2009). Expression of proteins in mammalian cells requires a very complex nutrient medium, sophisticated culture conditions and fermenter technology. These specific requirements make the overall protein production process very expensive. Apart from this, these cultures are very prone to virus and prions contamination (Bisbee 1993).

1.2.5 Transgenic animals

Transgenic animals technology is one of the most widely used technology for the production of heterologous / recombinant proteins. Generally mammary glands cells are targeted for the recombinant protein production in the dairy animals e.g. cow, sheep and goat etc. as they are able to produce milk for a longer period of time. Various other transgenic animals were also developed for the production of heterologous proteins such as mouse, rabbit etc (Rudolph 1997).

Therapeutic proteins produced in transgenic animals are tissue plasminogen activator (tPA) produced in the milk of transgenic goat (Ebert et al. 1991, Glanz 1992, Ebert et al. 1994), recombinant human Factor IX and human α-1-antitrypsin produced in transgenic sheep (Yull et al 1995; Carver et al 1992). Transgenic animal products are very much similar to the human proteins in the glycosylation pattern. These products are found safe and no adverse effect was observed on human health during clinical trials (McKown and Teutonico 1999). Further, human growth hormone was produced in the milk of transgenic rabbits (Lipinski et al. 2012) and human proinsulin was produced in milk of transgenic mice (Qian et al. 2014).

Transgenic animals as producer of the recombinant proteins provide significant quantity of proteins. However, the development of these animals is very time consuming procedure because particular time period is required for the growth of animal to reach up to the production stage of the proteins. So, in that way production of recombinant protein is not economic in transgenic animals as well.

1.2.6 Transgenic plants and plant cells

Plants are rising as viable and advantageous host systems for heterologous protein expression as they provide relatively high biomass and easy commercial scaleup with the requirement of very simple culture conditions and medium for growth and development. They have low risk of contamination and considered safe than the other available or conventional host systems (Doran 2000; Fischer et al. 2004; Sharma and Sharma 2009; Tremblay et al. 2010). Plants provide different forms of culture systems for expression of heterologous proteins such as callus culture, suspension culture, hairy root culture, organ culture etc (Sharma et al. 2014). First transgenic plant was developed in 1983 (Fraley et al. 1983). Further, many essential bioactive molecules were expressed in plants such as various peptides, vaccine antigens, antibodies, diagnostic proteins, nutritional supplements, enzymes and biodegradable plastics (Sharma and Sharma 2009).

1.3 Plant as a potential host systems

Plants as host systems have many advantages over the other available conventional expression system. This has been proven by successful overexpression of various heterologous proteins in plants such as vaccines, antibodies and therapeutic proteins etc. Many of these proteins are in clinical trials such as antibody CaroRx™ to prevent tooth decay and Idiotype vaccines (against follicular B cell lymphoma) were expressed in tobacco, Insulin was produced in safflower (Ma et al. 1998; McCormick et al. 2008; Boothe et al 2009). Successful expression and commercialization of Elelyso^{TM} (for the treatment of Gaucher's disease) in genetically engineered carrot cells is the major breakthrough study (Fox 2012) which has again proven the potential of plant as a host system. However, plants have very long way to go before it gets established as a potential host system due to specific limitation / problem associated to it.

1.3.1 Problem and strategic solutions

The major limitation of plants to be used as a host system of choice is low expression or production of proteins. Many strategies to overcome this limitation such as insertion of stronger promoter, codon optimization and organelle targeting etc. have been applied.

1.3.1.1 Strong promoter

Expression of a gene at its transcriptional level is driven by promoter. Promoter works as a regulatory element to drive the transcription of the gene. It contains the sequences which are required for RNA polymerase binding to start transcription. Various types of promoter like constitutive promoter, tissue-specific promoter and inducible promoter are often used to regulate the expression of gene with different activity, efficiency and pattern in plants.

a) Constitutive promoters

This type of promoter has the potency to express protein in all the part of plants. Resulting in higher expression of proteins. Regulation of gene expression by constitutive promoter is not affected by environmental factors and developmental stage of plants.

Example: Cauliflower mosaic virus 35S (CaMV 35S) promoter is a constitutive promoter, it confers high-level of gene expression in dicot plants (Odell et al. 1985, Gutierrez-Ortega et al. 2005). Ubiquitin is also a constitutive promoter which is being widely used in monocot plants (Christensen and Quail 1996).

b) Tissue-specific promoters

These promoters have the capacity to regulate gene expression in tissue or developmental stage-specific manner. So, the expression of gene, regulated by this promoter will be focused in the specific tissues of the plants which helps the protein to get accumulated in particular organ of plant.

Example: Patatin promoter is the potato tuber-specific promoter and can be used to concentrate the expression of protein in tubers (Jefferson et al. 1990).

Leaf specific promoters such as rbcS gene promoter was used to concentrate the expression of proteins in plant leaves (Dai et al. 2000; Golovkin et al. 2007).

Tissue specific expression of heterologous protein makes it downstream process easy.

c) Inducible Promoters

These types of promoters need some inducible compounds or factors such as biotic or abiotic factors to regulate their activity to promote gene expression. These kinds of promoters also lead to higher expression of heterologous proteins in plants.

Example: Chemical-inducible promoters: These promoters need some chemical inducers to drive the expression of foreign genes in plants such as alcohol, tetracycline, steroids etc. Induced expression of GFP was observed in transgenic *Nicotiana benthamiana* using a double-inducible viral vector containing ethanol inducing promoter (Werner et al. 2011)
Physically inducible promoters: The activity of these promoters is regulated by the environmental factors such as temperature, light, salt stress and desiccation etc. A sucrose starvation-inducible promoter of rice alpha amylase (αAmy3) gene has been used to express human interferon gamma in rice cell suspension cultures (Chen et al. 2004).

1.3.1.2 Codon usage or optimization

Expression of foreign gene in desired host is always not supported by host system because of the presence of specific codons in genes which may or may not present in host system. Resulting in reduced expression of gene in particular host system (Gustafssion et al. 2004). In protein-coding genes, synonymous codons that code for same amino acid do not appear at the same frequency. This kind of codon bias or difference in synonymous codon usage subsists in a wide variety of organisms from prokaryotes to eukaryotes. To overcome this, codon optimization technique is used which helps in up regulation of expression of heterologus proteins in particular host system.
Codon optimization of various genes was studied in various plants such as higher expression of codon optimized GFP was analysed in tobacco plant (Rouwendal et al. 1997). Synthetic cry1Ab gene was developed with optimised codons and expressed in the tobacco plastomes (Mirza and khan 2013).

1.3.1.3 Plant cell organelle targeting

Plant cell organelles targeting is also one of the strategies to increase expression of heterologous proteins in plants as it can accumulate protein in a specific organelle or organ

using signal peptide. In this strategy protein accumulation can be targeted to the endoplasmic reticulum, golgibody, hydrolytic compartments of the cell or secreted out of the cells.

a) Endoplasmic reticulum (ER)

Endoplasmic reticulum of plant cells is targeted for the higher accumulation of proteins. Retention of the heterologous proteins in plant cell requires a carboxy terminal tetrapeptide, such as KDEL or HDEL; it gets recognised by a receptor located in the golgi body and then proteins retrieved back into ER. It is found that addition of the signal peptide along with retention signal lead to the higher accumulation of the proteins in the ER (Gomord et al. 1997; Pelham 1990; Arakawa et al. 1998; Ko et al. 2003).

b) Plastids

Plastid is one of the major organelles of plant cell. This is the photosynthetic site used for the food production in plant cells. A plant cell contains large number of plastids with large number of plastid genome. Therefore, plastid transformation permits the maximum introduction of copies of transgenes per plant cell. It efficiently enhances the protein accumulation in the cell e.g. Higher expression of viral peptide animal vaccine was studied in transgenic protoplast of tobacco cells (Molina et al. 2004) and expression of *Bacillus thuringiensis* Cry1Ab protoxin was also observed in soybean plastid (Dufourmantel et al. 2005).

1.3.1.4 Intron-mediated enhancement (IME)

Intron-mediated enhancement is one of the important strategies to up regulate expression of heterologous proteins in plants. It needs insertion of specific intron within transgene at transcriptional region to regulate its expression. IME was studied in many plant cells using diverse introns for the expression of various heterologous proteins. IME strategy was found to increase transgene expression around 20 fold to more than 200 fold in plant cells (Mascarenhas et al. 1990; Akua et al. 2010). Many introns have been isolated from plants (native and non-native context) to regulate transgene expression in plants such as Adh1, Sh1, Ubi1, GapA1, SalT, Tpi, Waxy1, Cat1, AtMHX etc (Callis et al. 1987; Mascarenhas et al. 1990; Vasil et al. 1989; Vain et al, 1996; Donath et al., 1995; Rethmeier et al. 1997; Xu et al. 1994; Li et al 1995; Takumi et al 1994; Akua et al 2010). Many reporter proteins such as chloramphenicol acetyltransferase (CAT), β- glucuronidase (GUS), luciferase (LUC)

were successfully expressed in various plant cells (Callis et al. 1987; Mascarenhas et al. 1990; Kato et al. 1998; Tanaka et al. 1990; Akua et al. 2010; Bourdon et al. 2001).

In view of these reports and strategies regarding higher expression of heterologous protein in various hosts we have designed our study which deals with the overexpression of heterologous protein in plants using one of the potential strategy. For the study, erythropoietin was chosen as a protein to be expressed, *Nicotiana tabacum* plant as a host system and IME as strategy.

1.4 The project

This section deals with the details description of the use of Intron-mediated enhancement strategy to enhance the erythropoietin protein expression in transgenic *Nicotiana tabacum* plant.

1.4.1 IME: Upregulation strategy

In the present study, IME strategy was selected for the overexpression of erythropoietin in *N. tabacum.* Many studies have proven the strength of this strategy to upregulate the gene expression to a significant level (Mascarenhas et al. 1990; Akua et al. 2010). The first intron-dependent alcohol dehydrogenase (*Adh1)* gene expression study was performed in maize cells where the gene was expressed in the absence of its all 9 introns. Expression of *Adh1* gene was observed 50 to 100- fold less than intact gene construct which revealed that removal of intron from gene lead to decrease in expression of the gene. To analyse impact of intron on gene expression, only intron 1 was inserted back in to the gene and it was found that presence of intron 1 alone restored the level of the gene expression up to normal expression level of intact gene. The last two introns were also found to be stimulating the expression of *Adh1* gene, but expression level was around three fold lower than intact gene (Callis et al. 1987). The role of intron in gene expression was confirmed by subsequent study in 1990, where effect of maize Adh1 intron on expression of chloramphenicol acetyl transferase (CAT) gene in maize protoplast (Black Mexican Sweet) was studied. Adh1 intron 2 and intron 6 were individually inserted at different position in gene such as at 5′ UTR (untranslated region) and 3′ UTR. Expression level of CAT gene was enhanced by 12-fold with insertion of Adh1 introns 2 and 20-fold by insertion of Adh1 intron 6 in chimeric construct. This enhancement was observed with intron located at 5′ UTR of the gene but not with the one located within the 3' UTR of

the gene. This study revealed that only few specific introns can only lead to expression enhancement of the gene. This enhancement of gene expression phenomenon mediated by intron was first time named as intron-mediated enhancement in 1990 (Mascarenhas et al. 1990). Since then many studies have been performed to analyze this phenomenon in plants ranging from monocots to dicots. Plant introns that stimulate gene expression have been discovered in many plants such as petunia, blue grass, oat, rice, maize, castor bean, potato, arabidopsis, soybean and tobacco (Dean et al. 1989; Vain et al. 1996; Bruce and Quail 1990; McElroy et al. 1990; Snowden et al. 1996; Rethmeier et al. 1997; Tanaka et al. 1990; Leon et al. 1991; Fu et al. 1995a; Fu et al. 1995b; Rose and Last 1997; Chaubet-Gigot et al. 2001; Kato et al. 1998; Plesse et al. 2001).

As stated earlier, not all the introns have the potency to upregulate gene expression; hence, selection of appropriate intron becomes crucial for IME. In 2008, to analyse the potential of intron, a bioinformatics based software IMEter was developed, which recognises introns responsible for significant increase in gene expression. Results of this software were experimentally tested on *Arabidopsis* and *Oryza sativa* (Rose et al. 2008; Morello et al. 2011). In 2011, a study was carried out to investigate the sequence and signal of IME in the whole plant genome of many plant varieties and it was observed that IME signals are present in the direction of 5′ end of introns. IME signals are found to be conserved in various sequenced plant genomes as well as in numerous monocots, dicot plant species and also in lycophyte and moss (Parra et al. 2011).

IME strategy has the potential to work alone or in combination with other strategies such as insertion of strong promoter, codon usage and organelle targeting etc to regulate transgene expression (Zhang et al. 2007) **(Figure 1.2)**.

Figure 1.2: IME strategy can be used either alone or in combination with other existing strategies for enhancing gene expression.

1.4.1.1 Armaments of IME strategy

There is a series of specific requirements towards intron to make IME strategy successful **(Figure 1.3).** These requirements include-

- **a.** The size of the intron to be used
- **b.** The position of intron in the gene
- **c.** The conserved region of intron

These all specific features direct proper splicing of intron which ultimately leads to the expression enhancement of transgene (Rose 2002).

Figure 1.3: Specific features of an intron for the efficient splicing

a) Size of intron: Size of intron is one of the important criteria for IME. Proper size of intron can only allow the proper assembly of spliceosomal apparatus which leads to the mRNA accumulation. It was found that in case of monocot and dicot plants 70-73 nucleotides size is the minimum functional length of the intron for IME (Steitz et al. 1988; Goodall and Filipowicz 1990; Niu and Yang, 2011).

b) Position of intron: In IME, position of intron plays an important role in regulation of gene expression. Introns were found to be good for enhancing the expression of gene when present at start of transcriptional region of gene (Callis et al. 1987; Mascarenhas et al. 1990; Clancy et al. 1994; Donath et al. 1995; Parra et al. 2011). Insertion of intron towards 5′ end of gene allow the higher expression of gene whereas shift towards the 3′ end lead to the decrease in the gene expression. Introns present in close proximity to start of transcription site are generally rich in IME signals than distal introns (Mascarenhas et al. 1990; Callis et

al. 1987). Some positions of intron in gene was found good for higher gene expression such as within the coding region, between the promoter region and the coding sequences, and within the 5’ UTR (Tanaka et al. 1990; Bourdon et al. 2001). It was observed that first intron present in the 5' UTR of eukaryotic gene of different species is generally longer than other introns present in the coding region and 3’ UTR of the gene. These longer first introns are found to be more efficient and are capable to regulate the expression level of gene. For example, first intron of maize Adh1, Sh1, Ubi1, GapA1 (Callis et al. 1987; Vasil et al. 1989; Mascarenhas et al. 1990; Donath et al. 1995; Vain et al. 1996).

c) Conserved region

Introns have universal conserved region such as GU dinucleotides at the 5’ splice site and AG dinucleotides at the 3' splice site. Apart from this, introns from different species have different conserved regions which make the introns recognizable for splicing by splicing apparatus, such as in yeast UACUAAC is conserved sequence (Padgett et al. 1986), vertebrates have polypyrimidine tract towards the 3’ splice site which gets recognized by splicing apparatus. In case of plant species, composition of nucleotides of introns varies. GC rich nucleotide intron sequence is preferable in monocots while AU rich composition is preferable in dicots for splicing **(Figure 1.4).** Change in nucleotide composition of intron will prevent its splicing in plant cells (Goodall and Filipowicz 1991).

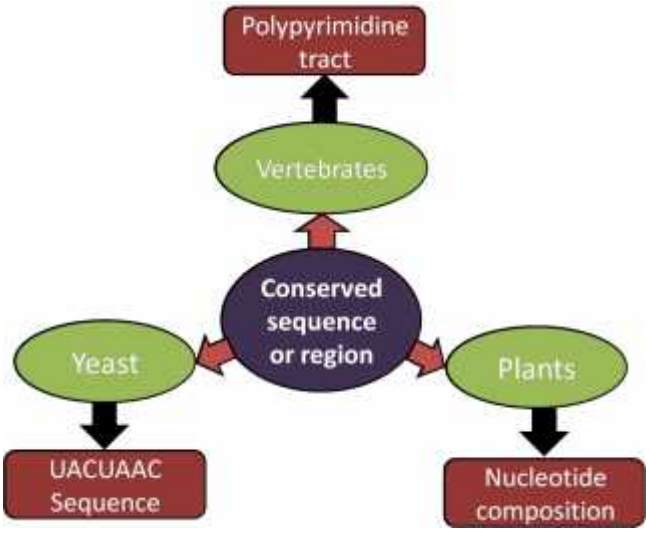


Figure 1.4: Conserved region in intron sequences of different species

1.4.1.2 Synthetic7 intron

In the present study, to make IME strategy successful for overexpression of protein in plant, '**synthetic7**' (syn7) intron was used. As the name suggest Syn7 intron is a designed and synthesized intron (Synthetic intron), for up regulation of gene expression. This intron was found efficient to get spliced off properly during mRNA processing in monocot as well as dicot plants which lead to over expression of gene (Goodall and Filipowicz 1989; Hir et al. 2003). Based on positive reports of previous studies, Syn7 intron was selected for the regulation of expression of erythropoietin in *N. tabacum* plant.

As described earlier it is a synthetic intron which was designed in 1989. It has 75% AT- rich nucleotides and 85 bp in size. It was designed to analyse the requirement of intron for its proper splicing in the plant cells by spliceosome machinery. Splicing involves the recognition of conserved regions in the intron by spliceosome machinery. As plants do not have any conserved region in their intron, the spliceosome machinery recognises specific nucleotide composition based on dicot and monocot plants. It was found that AU rich nucleotide introns were found compatible for processing in dicot plant cells. On that basis a synthetic intron was constructed with high quantity of the A and U nucleotides and universal splice sites GU dinucleotide present at the 5′ end and AG dinucleotide at 3′ end of the intron. Syn7 was tested in protoplast cells of the *Nicotiana plumbaginifolia* where it's efficient splicing was observed (Goodall and Filipowicz 1989, Goodall and Filipowicz 1991).

1.4.2 Erythropoietin (EPO): Heterologous protein

Erythropoietin is a cytokine hormone of human origin; it stimulates the production of red blood cells and protects these cells from apoptosis. Red blood cells are produced due to the erythropoiesis which occurs in kidney (adult) and liver (embryo or fetal). Kidney cells are sensitive to low oxygen level in blood which is known as hypoxia. In response to hypoxia, kidney cells produce erythropoietin protein which stimulates the bone marrow to produce more red cells to increase the oxygen-carrying capacity of the blood. Erythropoietin is expressed or produced by the *EPO* gene, which is located on human chromosome 7 between 7q11-7q22 (Jelkmann et al. 2008). It has 4 introns and 5 exons. Post-transcriptionally, it produces single mature transcript of 1330 base pairs and is finally translated into a polypeptide chain containing 193 amino acids precursor. During the posttranscriptional

modification, two disulfide bonds are formed between Cys-7 and Cys-161 and between Cys-29 and Cys-33, and this process takes place along with the removal of an N-terminal hydrophobic secretory sequence of 27 amino acids. It is thought that Arg-166 in C-terminal portion of EPO protein is cleaved off before its release into circulation. Therefore, the primary structure of this mature protein contains only 165 amino acids. Secreted erythropoietin is a highly glycosylated protein having molecular weight of 34 kDa. Glycosylation occurs with the addition of N-linked oligosaccharides to Asn-24, Asn-38, and Asn-83; and an acidic O-linked oligosaccharide to Ser-126 during the post transcriptional modification **(Figure 1.5)**.

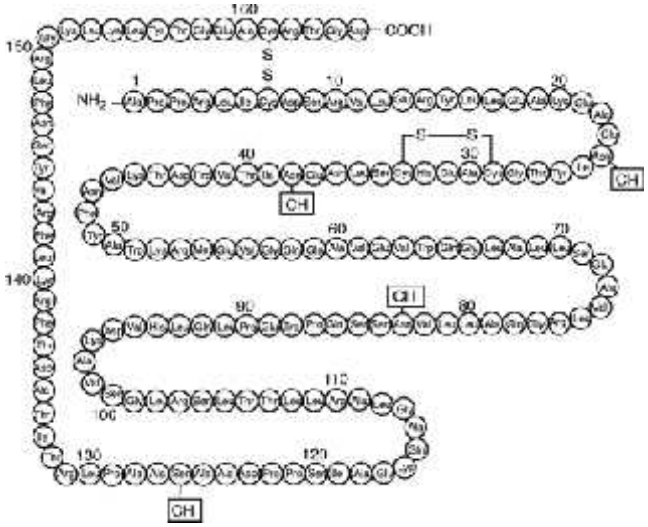


Figure 1.5: Primary structure of erythropoietin
Reference: (Lappin 2003)

40% of the molecular mass of EPO is covered by the oligosaccharide (carbohydrate) chains. The molecular weight of non-glycosylated EPO was estimated 18 kD and for glycosylated forms 30-34 kDa. The carbohydrate chains of EPO are mainly responsible for its integrity and stability. N-oligosaccharide chains prevent EPO from renal clearance. So, these both terminal chains are required for *in vivo* activity of erythropoietin (Jelkmann 2003). The half-life ($t_{1/2}$) of EPO is ~5 hours (Eckardt et al. 1989), which requires an average EPO production rate of ~2 U/kg/day. In cases of severe anemia, circulating EPO levels can increase up to 1000- fold because of a logarithmic increase in the number of cells producing EPO (Erslev 1991; Koury et al. 1989).

In 1989, first time recombinant erythropoietin Epoetin alfa was produced in Chinese hamster ovary (CHO) cell line transfected with human EPO cDNA. Nowadays many different and potential isoforms of EPO are available worldwide for the therapy purpose (Reichel and Gmeiner 2010). Erythropoietin is one of the leading products in the biopharmaceutical market. It covers the maximum share of the recombinant protein market. This is used for treatment of many common diseases such as anaemia, kidney failure and cancer (Hodges et al. 2007; Nangaku et al 2006; Morais et al. 2013).

Demand of erythropoietin is increasing 20% every year. The global sale of erythropoietin was around $13 billion which was expected to grow around $701 billion till 2010 (Jo and Yann 2006). High market value represents the high demand of this protein all over the world for the therapeutic purpose. At present erythropoietin available in the market is produced in mammalian cells to generate high-quality proteins that are similar in their biochemical properties to the naturally occurring human forms.

1.4.3 *Nicotiana tabacum*: Plant host system

The common name of *Nicotiana tabacum* is tobacco. It is the member of Solanaceae family and basically, originated from the Tropical America. It is a perennial herbaceous leafy crop **(Figure 1.6)** which gives high biomass and rapid scalability in short time period**.** It grows to heights between 1 to 2 metres. This plant is natural allotetraploid (2n=4x=48) formed between the hybridization of two diploid (2n=24) progenitors *Nicotianan sylvestris* and *Nicotiana tomentosiformis* (Rao and Stokes 1963). The genome size of *N. tabacum* is 4434 Mb.

Figure 1.6: Field grown plant of *N. tabacum*

The well-known genome information and other characteristics make the plant extremely versatile and good model system for all aspects of tissue culture and genetic engineering studies. Due to these reasons it is known as white mouse in the field of plant biotechnology (Tremblay et al. 2010).

From the establishment of the first transgenic plant of *N. tabacum* in 1983 to till date, various therapeutic proteins such as human somatotropin, erythropoietin, human epidermal growth factor, avidin and streptavidin (Staub et al. 2000, Matsumoto et al. 1995, Higo et al. 1993, Murray et al. 2002); vaccine antigens such as hepatitis B virus surface antigen (HBsAg), cholera toxinsubunits A and B, measles virus hemagglutinin H protein (Mason et al. 1992, Hein et al. 1996, Huang et al. 2001) and antibodies such as single chain immunotoxin composed of bryodin 1 fused to scFv region of anti-CD 40 antibody G28-5, scFv of IgG from mouse B cells lymphoma, T48.66/GS8 diabody (Francisco et al. 1997; McCormick et al. 1999; Vaquero et al. 2002) were successfully expressed in *Nicotiana tabacum* plant.

In addition, successful large scale production of *N. tabacum* as a crop makes it suitable for the commercial production of recombinant proteins. Containment of *N. tabacum* is easier than the other plants because it is a self-pollinated plant. It is neither a food nor a feed crop. So, there is little risk of food and feed chain contamination (Ma et al. 2003).

1.5 Objectives

The project was focused to enhance the expression of erythropoietin protein in *N. tabacum* plant using intron-mediated enhancement strategy and was entitled as **'Intron mediated enhancement of heterologous protein in plant'**. To accomplish project successfully following objectives were set.

1. **Maintenance of *in vitro* culture of *Nicotiana tabacum Wisconsin V 38.***
2. **Vector desigining and construction**
    - *In silico work for vector and primer designing*
    - *Procurement of intron sequence*
    - *Restriction digestion and ligation for insertion of desired transgenes*
    - *Confirmation of constructed vectors by sequencing*
3. **Bacterial transformation**
    - *Transformation of E. coli cells with vector containing desired gene*
    - *Transformation of Agrobacterium with expression vector (Electroporation)*
    - *Analysis of transgene*
4. **Plant genetic transformation**
    - *Transformation of N. tabacum with control vector and test expression vector*
    - *Selection of transformants*
    - *Shoot regeneration*
    - *Analysis of transformants*
    - *Root regeneration*
    - *Hardening of transgenic plants*
5. **Analysis of heterologous protein expression**
    - *Analysis of mRNA expression using Real Time PCR*
    - *Analysis of expressed protein by ELISA assay*

Chapter 2

Review of literature

2.1 Intron and its role in gene regulation

Introns were discovered in 1970s. They were found in eukaryotes as a part of genomic DNA. Earlier introns were considered as a non-functional element of genomic DNA as they get spliced off during mRNA processing and were not involved in protein coding process (Jeffares et al. 2006; Roy and Gilbert 2006; Belshaw and Bansasson 2006). However, introns are no more treated as nonsense sequences as many studies revealed it's potential in regulation of gene / transgene expression. Earlier studies in this regard was conducted in mammalian cells and observed importance of intron in regulation of the gene expression. Involvement of intron within gene followed by splicing of intron, mRNA processing and nucleus export, all these steps are interconnected and were found responsible for the enhancement of gene expression. Presence of intron and other nuclear and cytoplasmic processing were also studied in mammalian cells for better understanding of presence of intron in gene and its impact on regulation of gene expression (Gruss et al. 1979; Gasser et al. 1982; Luo and Reed 1999; Hir et al. 2003). Intron dependent gene expression was widening up to plant cells after initial studies in mammalian cells.

2.1.1 Intron regulated studies in mammalian cells

Effect of intron on gene expression was studied in mammalian cells and was found to be linked with gene expression. In late 1970s, a study was carried out in which simian virus 40 deletion mutants were constructed lacking specifically intron sequences for a late viral mRNA. The construct was prepared by replacing the late simian virus 40 genes with DNA segment from reverse transcription of the viral mRNAs. It was observed that deletion of intron sequences from this mutant prevented the expression of major capsid protein VP1. This failure revealed a defect in the posttranscriptional processing of the viral RNA which indicates that splicing is an essential function in the biogenesis of certain mRNAs (Gruss et al. 1979). In another study, dihydrofolate reductase (DHFR) gene constructs were prepared. In

two of the genes constructs, first two introns of DHFR gene were present while other three constructs were devoid of introns. DHFR deficient Chinese hamster ovary cells were transfected with these constructs and observed for expression of DHFR gene. Expression of DHFR gene in the cells was found to be higher with the construct having intron. Transfection frequencies of DHFR gene was also observed high in the construct having introns. In the study, it was assumed that this difference could be the result of the presence or absence of introns (Gasser et al. 1982). Further, effect of intron on gene expression was studied in transgenic mice also. In this study, chloramphenicol acetyltransferase (CAT) gene was expressed with and without an intron under the control of human histone H4 promoter. Expression of CAT was found 5-300-fold higher in transgene harbouring an intron construct (Choi et al. 1991). In 2003, effect of intron on gene expression was studied in HeLa cells. The sixth intron from the human triose phosphate isomerase (TPI) gene was used to analyse the expression of Renilla luciferase gene in HeLa cells. Intron was inserted within the open reading frame of the gene at different positions such as at the 5′ and 3′ end of the gene. Significantly increased gene expression was observed with both the intron positon. However, gene expression was around 19 fold higher with 5′ intron as compared with 3′ intron construct. For both the positions, stimulation of protein expression was largely due to increased mRNA accumulation and translational yield (Nott et al. 2003). All these studies confirmed the significance of presence of intron in expression of gene.

2.1.2 Intron regulated studies in plant cells

In plants in late 1980s, a study was carried out where the importance of intron was observed when removal of all the introns from alcohol dehydrogenase gene (*Adh1*) was resulted in 100 fold drop in expression of gene and presence of just one intron was found enough to bring the expression up to normal level (Callis et al. 1987). Effect of intron was further studied in maize cells and higher expression of CAT gene was observed in the presence of intron (Mascarenhas et al. 1990). It was also noticed that every intron cannot work as a regulatory element. It requires some specific features to enhance the expression of gene in plants such as size, position and nucleotide composition etc (Rose 2002). In 2008, a software was developed with the help of bioinformatics tools and approaches to determine the appropriate intron on the basis of positive or negative scores. This software scores the intron on the basis of required features which are important to lead the gene expression enhancement (Rose 2008).

2.2 Link between intron splicing and mRNA export

On the basis of previous studies, a link was found between splicing of intron and mRNA export which finally lead to regulated gene expression. This link was initially studied in mammalian cells and further tried to understand in plant cells also.

2.2.1 In mammalian cells

Presence of introns in gene leads to the splicing of intron from mRNA and then export of mRNA from nucleus to cytoplasm. Splicing is one of the important steps in mRNA processing which has been found to be linked with up regulation of expression of gene. This link was confirmed or analysed in many studies. In late 1990s, relation between intron splicing and mRNA export was observed in *Xenopus oocytes*. In this study two pre-mRNAs with or without an intron were injected into *Xenopus oocyte* nuclei. It was observed that intron spliced mRNAs have been exported much more rapidly and efficiently than the mRNAs lacking intron. The study revealed that splicing of intron generates a specific nucleoprotein complex that targets mRNA for export which finally leads to the higher accumulation of mRNA (Luo and Reed 1999). Further, a study was conducted to investigate whether splicing promotes mRNA export in mammalian cells or not. HeLa cells were transiently transfected with intron-containing and intronless constructs of β- globin DNA. It was observed that spliced β- globin mRNA was largely cytoplasmic whereas its cDNA transcript was mostly retained in nucleus. The association between splicing and mRNA export in mammalian cells was analysed by using FISH and the results indicated that the efficiency of mRNA export is enhanced 6- to 10- fold, thus confirming that splicing promotes mRNA export in mammalian cells and the functional coupling between splicing and mRNA export is a conserved feature of gene expression in higher eukaryotes (Valencia et al. 2008).

2.2.2 In plant cells

Further, importance of splicing was studied in plant cells. Splicing is one of the most contentious issues in plant cells for gene expression. In some studies, splicing was found very crucial for the up regulation of gene expression, a study was performed where it was observed that the first intron of maize *Adh1* gene contains some motifs which was vital for the intron splicing, as deletion of a large part of intron sequence prevented its splicing due to the

removal of essential motifs (Luehrsen and Walbot 1994). In another study, it was found that mutation at the intron splice sites blocks the splicing of the intron and reduces the gene expression also e.g. Sh1 first intron has the capacity to enhance the CAT gene expression in maize cells around 20-50-folds with the efficient splicing of the Sh1 intron. A mutation at the intron splice sites of Sh1 intron block the splicing of intron and reduce expression to about 2-fold (Clancy and Hannah 2002). AtMHX promoter alone was also found unable to increase the expression of GUS in *Arabidopsis thaliana* in the absence of its leader intron and its splicing. These examples suggest, presence of intron is essential for enhanced gene expression (Akua et al. 2010). However, some studies revealed that splicing itself is not sufficient to enhance expression of gene e.g. in a previous study it was found that only splicing itself is not enough for gene expression enhancement in *Arabidopsis thaliana*, it can be affected by some other factors also such as mutation at 5′ splice site or 3′ splice site, elimination of branch point, reduction of U contents and improper assembly of spliceosome machinery on intron. (Rose 2002).

The above mentioned studies in animal and plant cells suggest that in most of the cases inclusion of intron enhances the expression of gene/transgene. In plant cells, the regulatory role of intron in gene expression was first time named as intron-mediated enhancement (IME) in 1990 (Mascarenhas et al. 1990). This study was further extended to various other plants for the expression of different heterologous proteins.

2.3 IME in plants

In last 25 years, many studies were performed to analyse the effect of intron of the regulation of gene expression in plants. Various heterologous proteins such as reporter and foreign proteins were expressed successfully using many introns and significant level of expression was observed in plants. Details of the studies were given in **Table 2.1**.

Table 2.1: Brief on different plant introns and reporter genes used in IME						
Sr. No.	**Intron**	**Source**	**Reporter Gene**	**Host (Cells)**	**Fold increase in expression level (%)**	**References**
1	*RpoT*-i4	Maize	*LUC*	Maize (BMS)	2.4 fold	Bourdon et al. 2001
2	*PEPC* Intron1	Soybean	*GUS*	Tobacco, Rice	3 fold	Kato et al. 1998
3	Adh-1	Maize	*CAT*	Maize	100 fold	Callis et al. 1987
4	*Cat*-1	Castor	*GUS*	Rice protoplast Rice tissues	10-40 fold 80-90 fold	Tanaka et al. 1990
5	*Sh*-1	Maize	*CAT*	Maize	20-50 fold	Clancy and Hannah 2002
6	*Ubi*-1	Maize	*GUS*	Maize Blue grass	71 fold 26 fold	Vain et al. 1996
7	*Adh*-2 &6	Maize	*CAT*	Maize	12-20 fold	Mascarenhas et al. 1990
8	*AtMHX* Leader intron	*Arabidopsis*	*GUS*	*Arabidopsis*	3-272 fold	Akua et al. 2010
9	*RpoT*-i4 & *UBQ*10-i1	Maize and *Arabidopsis*	*Luciferase*	Barley	1.25 fold 2.87 fold	Bartlett et al. 2009
10	*Sal*T	Rice	*Cat and bar*	Maize	10-30 fold	Rethmeier et al. 1997
11	*Cat* 1	Castor bean	*uidA*	Wheat	3-5 fold	Takumi et al. 1994
12	*Waxy*1	Rice	*GUS*	Rice	15 fold	Li et al. 1995
13	*OsTub*6 Leader intron	Rice	*GUS*	Rice	10 fold	Giani et al. 2009
14	*SOD*1	Rice	*GUS* *LUC* *LUC* *RLUC*	Rice seedlings Rice Wheat seedlings Maize seedlings	16.9- 19.5 fold 4.9- 27.7 fold 7.6 fold 6.5 fold	Morita et al. 2012
15	*ADF1*	Petunia Arabidopsis	*GUS*	*Arabidopsis thaliana*	4-8 fold	Jeong et al. 2007

2.3.1 Therapeutic protein expression in plants using IME

IME strategy was also extended to regulate the expression of therapeutic proteins in plants. Expression of erythropoietin protein was studied in mutant cell lines of moss (*Physcomitrella patens*). In this study, plant-specific α 1,3-fucosyltransferase and β 1,2-xylosyltransferase genes have been knocked out to prevent the addition of non-essential plant glycan to the expressed protein. Moss specific vector and its own intron were used to enhanced the expression of erythropoietin in moss. There was higher expression of recombinant human erythropoietin (rhEPO) at level of 250 µg/g of dry weight of moss material after 6 days obtained (Weise et al. 2007). In another study, expression of human coagulation factor IX (hFIX) in fruit of tomato (*Lycopersicon esculentum)* was studied using its own intron I of hFIX and polygalacturonase (PG) promoter (fruit specific promoter). Successful expression of hFIX protein was observed in mature fruits of tomato (Zhang et al. 2007). These studies further strengthen the concept of use of intron for successful expression of the transgene (therapeutic proteins).

2.4 Genesis of the project

In view of successful studies on intron mediated enhancement of various reporter gene (Table 2.1) and therapeutic protein, the present study was designed to apply IME strategy in therapeutic protein expression in higher plants. Higher plants have potential to serve as an economic (Ahmad et al. 2012) and bulk production (biofactories) system if successful in high level of expression of biologically active therapeutic protein, present study was designed with the aim of high level of expression of **erythropoietin** – a human protein used as therapeutic protein in human ailments, in ***Nicotiana tabacum*** plant using **Synthetic7** (Syn7) **intron**. As earlier studies in this regard was suffered with compromised level of protein expression.

- **Selection of syn7 intron:** Intron syn 7 was selected for the study based on its efficient splicing in dicot plants as splicing is one of the crucial steps of mRNA processing to increase the level of expression (Goodall and Filipowicz 1990).
- **Selection of erythropoietin:** Erythropoietin is selected for the study as it is one of the leading therapeutic proteins in biopharmaceutical market. It is a human protein. It promotes red blood cell production in human body and used for the treatment of various

anaemia ailment. Available recombinant erythropoietin in market is usually produced in mammalian cell culture (Bisbee 1993). Another major region behind selection of this protein for the study is ongoing previous studies on this protein in our laboratory (Desai 2009).

- **Selection of *N. tabacum*:** This plant is being used as a model system or host for the expression of heterologous proteins as it is a well characterized plant and apt to tissue culture and genetic transformation techniques. It has been extensively explored for the expression of various therapeutic proteins (Matsumoto et al. 1993, 1995; Cheon et al. 2004; Sperb et al. 2011; Jez et al. 2013). Ongoing and previous studies on this plant were also one of the reasons to select this plant.

Previous laboratory work

Agrobacterium-mediated genetic transformation protocol was standardized for the expression of erythropoietin proteins in potato tuber of Indian tetraploid potato variety *Kufri Bahar* using patatin promoter (tuber specific). Polymerase chain reaction (PCR) analysis was performed to confirm the integration of the erythropoietin gene in potato plant using specific primers (Desai and Padh 2012). *In vitro* cultures of *N. tabacum* were also maintained in laboratory for various studies.

Present study

On the basis of previous laboratory work and all the previous literatures regarding the over expression of EPO in *N. tabacum* plants, it was observed that insertion of strong promoter itself is not enough to increase the expression of EPO in *N. tabacum*. According to literature survey, IME strategy was found efficient for the over expression of various proteins in plant. Therefore, in the present project IME strategy was used for the enhanced expression of the erythropoietin in *N. tabacum* plant. In IME strategy, Syn7 intron has been used to regulate the expression level of EPO as Syn7 was found suitable to process in both monocot and dicot plants by spliceosome machinery (Goodall and Filipowicz 1989). Proper intron processing by spliceosomal machinery is essential to leads for the enhanced expression of gene.

This project is a contribution to evaluate the role of IME strategy for the upregulation of expression of therapeutic protein. The results of the project will provide more information and understanding to explore this strategy further.

Chapter 3

Materials and Methods

This chapter of the thesis details the materials including chemicals, fine chemicals, solvents, kits used in the study. It also describes the instruments / equipments, methodologies or protocols used to carry out the experiments and to prove the hypothesis.

3.1 Materials

3.1.1 Chemicals and Reagents

Agarose, CTAB, Tris Base, Ethylenediaminetetraacetic acid disodium salt (EDTA), Glucose, Calcium chloride, Potassium acetate, Sodium acetate, Potassium chloride, Magnesium chloride, Trypton, Yeast extract, Sodium chloride, Luria agar, Luria broth, Phenol, Chloroform, Glycerol, Ethidium bromide, Bromophenol blue, Absolute Alcohol, Isopropanol, Sodium hydroxide, Glacial acetic acid, Sodium dodecyl sulphate, 8-hydroxyquinoline, Taq Buffers, Power SYBR green master mix, Deoxynucleotide triphosphate (dNTPs), $MgCl_2$ solution and Restriction digestion buffers are the reagents and chemicals used for the study..
All the chemical and reagents were of analytical grade and were procured from Sigma (India), HiMedia (India), s.d. fine (India), Qualigens (India), Merck (India), Rankem (India), Sisco laboratories (India), as per availability and requirement.

3.1.2 Experimental kit

Following experimental kits were used for the study.
Gel Elute^{TM} Extraction Kit (Sigma), RNeasy plant RNA kit (Qiagen), DIG DNA labelling and detection kit (Roche), Sybr Green Master Mix (ABI), Iscript^{TM} reverse transcription supermix.

3.1.3 Plant growth regulators

All plant growth regulators namely 6-Benzylaminopurine, Indole-3-acetic acid, 1-Naphthaleneacetic acid, Kinetin, Folic acid were procured from Merck (India) and HiMedia (India).

3.1.4 Antibiotics and standards

Antibiotics used in the experimental work i.e. Kanamycin, ampicillin and rifampicin were procured from Himedia, India. Cefotaxime (Alkem, India) was procured from the local pharmacy shop (S.A.L. Pharmacy, Ahmedabad, Gujarat, India), Basta® (Glufosinate Ammonium) was procured from Bayer CropScience Limited, Ahmedabad, Erythropoietin standard (Zyrop injection vial, 2000IU, Zydus, India) used for the ELISA assay was procured from S.A.L. Pharmacy, Ahmedabad, Gujarat, India. Nicotine standard (Sigma, India) used in the HPLC study was procured from Sigma.

3.1.5. Antibody and substrate

Antibodies used in the ELISA assay i.e. Primary antibody of erythropoietin and Secondary antibody (Anti goat anti rabit antibody) ALP conjugated were gifted from Intas Biopharmaceticals, Ahmedabad, Gujarat, India. TMB substrate used in the ELISA assay was procured from Sigma, India.

3.1.6. Enzymes

All the enzymes which include Nucleases (RNase), Taq DNA polymerase, ligase and restriction endonuclease enzymes *viz. Asc*I, *Avr*II, *BstE*II, *Hpa*I, *Nco*I *and Eco*RI were procured from Fermentas, India.

3.1.7. DNA Ruler

DNA ladders i.e. λ-DNA ladder digested with *Hind* III and GeneRuler 50bp DNA ladder were procured from Fermentas, India.

3.1.8. Instruments

The instruments which were used during study period were Laminar air flow (Polaris 1504, India; Klenzeids, India), Autoclave (Mediquip, India), Gel electrophoresis unit and Power supply (Biorad, USA), Gel documentation system (Biorad, USA), Table top microcentrifuge (Legend Micro 21, Thermo Fischer, India), Centrifuge Universal 320 R and Rotina 380 R (Hettich, Germany), Spectrophotometer UV-2450 (Shimadzu, Japan), Elisa Plate Reader (BIOTEK ELx800-MS), Electroporator (Gene Pulsar Xcell, Biorad, USA), Orbital Shaker (LETT, Orbitek, India), PCR Thermal cycler (Eppendorf, Germany), Real-time PCR Stepone (ABI, India), HPLC (LC-2010C_{HT} Shimadzu), Electronic balance (Mettler AE163, USA), pH meter (CL 46 Plus, Toshcon, India), Vortex (Eltek VM301, India), Water bath (YSI414, Yorco, India), Deep freeze (-20^{o}C) (Siemens, Germany), Digital camera (Sony, India), Hot Air Ovan (Cintex, India), Microwave (LG, India). Magnetic Stirrer (DIGIMAG M 2 D, Eltek, India), -80^{o}C Refrigerator (Anjelantoni, Italy).

3.1.9 Plastic wares

Following plastic wares were used for the study.

Beakers (Tarsons, India), Centrifuge tubes (15 ml) (Tarsons, India), Micro centrifuge tubes - 0.5 ml, 1.5 ml, 2.0 ml (Tarsons, India), 0.2 ml PCR tubes (Axygen, USA), Micro tips - 0.5-10 µl, 20-200 µl, 200-1000 µl (Tarsons, India), Fast optical 48-well reaction plate (0.1 ml) (ABI, India), Optical 8-cap strips (ABI, India), Elisa plate (Nunc, Thermo Scientific, India).

3.1.10 Glass wares

Wide mouth 150 ml and 250 ml culture flask, beaker, measuring cylinder, petriplates, pasteur pipette, glass pipette, volumetric flask, reagent bottle, glass rod, HPLC sample vial. All the glass wares used were procured from Borosil, Durasil (India) and Schott duran (Germany) companies.

3.1.11 Apparatus

Following apparatus were used for the study.

HPLC column (TOSOH TSK-GEL), Micropipettes of variable volumes (1000, 200, 20, 2 µl) (Thermo Scientific, India; Gilson, USA), scalpel, forceps, spatula, pestle mortar, magnetic magnetic stirrer beads (from local supplier).

3.1.12 Miscellaneous materials

Other miscellaneous materials used for the study were cotton, tissue paper, butter paper, 0.2 µm whatman filter papers, rubber bands and all these were procured from local vendors. 0.22 µm membrane filter (Pall India Pvt. Ltd., India), 0.22 µm filters (Merck Millipore, Germany).

3.1.13 Softwares

Following softwares were used for the present study, primer designing software i.e. Primer3 software V 4.0.0, Primer express V 3.0 for primer designing. New England Biolabs (NEB) cutter V 2.0 software was used to identify the restriction sites in vectors or DNA sequences. Multiple sequence alignment ClustalW 2 software was used for the study.

Statistical softwares i.e. MS Excel and Prism 5 were used for the data analysis in present study.

3.1.14 Data base

National Center for Biotechnology Information (NCBI), ENSEMBL data bases were used to procure the sequences and to blast the query sequence against database sequences.

3.1.15 Biologicals materials

Plant- *Nicotiana tabacum* L. Wisconsin-38 was used for the present study as a host system.

Bacterial cultures- *Escherichia coli* DH5α, *Agrobacterium tumefaciens* LBA 4404 which were used for the study was gifted by Prof. Stanton B. Gelvin from Purdue University, Indiana.

3.1.16 Vectors

Following three vectors were used for the present study.

Backbone vector pFGC5941 was procured from Arabidopsis Biological Resource Centre, USA.

Vector pPERDB33cEPO was gifted by Dr. Priti Desai from B. V. Patel PERD Centre, Ahmedabad, Gujarat, India.

Intron Syn7 after synthesis was delivered in vector pUC57 from GenScript, USA.

3.2 Methods

3.2.1 *In silico* work

a) Designing of intron and primers

- Some basic softwares such as New England Biolabs (NEB) cutter V 2.0 software was used during analysis and designing of intron Syn7 with required RE sites. All the essential analysis of vector construction and detection of RE sites in the vector were also performed using same software.
- Primer3 and OligoEvaluator^{TM} were used for the primer designing and analysis.

3.2.2 Molecular biology work

This section details the methods which have been used for the maintenance of bacterial culture, construction and cloning of vectors.

3.2.2.1 Growth and maintenance of bacterial cultures

a) ***Prepararion of luria broth medium:*** To prepare 10 ml medium of luria broth, 0.2g of luria broth powder was weighed and taken in the beaker. Milli Q water (8 ml) was added in beaker and dissolved properly. Final volume of medium was made up to 10 ml with milli Q water in measuring cylinder. Medium was poured in conical flask and closed with the cotton plug. Medium was sterilized and stored at room temperature for further use.

b) ***Preparation of luria agar medium:*** To prepare 50 ml medium of luria agar, 1.75 g of luria agar was weighed and put in 250 ml of conical flask. Milli Q (50 ml) water was added in flasks and closed with the cotton plug. Medium was sterilized and poured in petriplates allowed to solidify.

c) ***Revival and maintenance of E. coli DH5α culture from stab culture and glycerol stock:*** *E.coli* culture was revived from stab culture maintained in the laboratory by touching or inserting the microtip in the stab and that tip was put in 10 ml luria broth (LB) medium. (For culture revival from glycerol stock, it was inoculated in 10 ml LB medium. Inoculated culture flasks were incubated at 37^{o}C for 16 h at 180 rpm on shaker for growth. Further, cultures were maintained by regular subculture at regular intervals. Transformed *E. coli* DH5α cells were maintained with the appropriate antibiotics required for the maintenance of the plasmid DNA.

d) Revival and maintenance of A. tumefaciens LBA4404 bacterial culture from glycerol stock: Bacterial culture was revived from the glycerol stock stored at -20^{o}C. LB medium (10 ml) was taken and glycerol stock culture was added in to LB medium containing rifampicin antibiotic. Culture flask was incubated at 28^{o}C on shaker at 200 rpm for 16 h for growth. Further, maintenance of the culture was carried out at regular intervals. Transformed *A. tumefaciens* LBA4404 cells were maintained with the appropriate antibiotics required for the maintenance of the plasmid DNA (the vector).

e) Preservation and storage of bacterial culture: Sixteen hours freshly grown bacterial culture (800 µl) was taken in vial and glycerol (200 µl) was added in to that and mixed properly by inverting the tube. Glycerol stock vials were stored at -20^{o}C for future use.

3.2.2.2 Isolation of plasmid DNA using alkaline lysis method (Sambrook and Russell 2007)

After revival (section 3.2.2.1c) of bacterial culture, the overnight grown bacterial culture (1.5 ml) was taken in microfuge tubes (2 ml) and kept on ice for 10 min. These tubes were then centrifuged at 6000 rpm for 10 min at 4ºC, supernatant was decanted and pellet was gently resuspended in to 100 µl of pre-cooled ALS-I mix and incubated on ice for 5 min. To this 200 µl of freshly prepared ALS-II mix was added and gently mixed the cells with solution by inverting the tubes and incubated on ice for 7 min. Then 150 µl of pre-cooled ALS-III mix was added and mixed gently by inverting the tubes and incubated on ice for 10 min. These tubes were then centrifuged at 12000 rpm at 4ºC for 10 min. Supernatant was taken into fresh 1.5 ml microfuge tubes and 0.6 volume of isopropanol was added to the tube containing supernatant and these tubes were then incubated overnight at -20ºC. after overnight incubation these tubes were centrifuge at 12000 rpm for 20 min at 4ºc. Supernatant was decanted and the

pellet was treated with 70% alcohol and kept at room temperature (RT) for 10 min. These tubes were again centrifuged at 12000 rpm for 10 min at RT. Supernatant was decanted and the pellet was air dried. In the final step pellet was dissolved into 50 µl of Tris-EDTA, pH 8.0 solution containing RNase (concentration of RNase), and incubated overnight at 37ºC.
Composition and Preparation of ALS I, II, III is mentioned in ***Appendix I.***

3.2.2.3 Phenol-chloroform purification (Sambrook and Russell 2007)

The isolated plasmid DNA was subjected to phenol chloroform purification method.
Plasmid DNA sample (section 3.2.2.2) 0.5 µl was taken in 1.5 ml microfuge tubes and equal volume of equilibrated phenol -chloroform (1:1) was added and mixed properly. Tubes were centrifuged at 12000 rpm for 5 min at 24ºC. Upper phase was transferred in to fresh microfuge tubes and equal volume of chloroform-isoamyl alcohol was mixed and centrifuged at 12000 rpm for 5 min at 24ºC. Upper layer from these tubes were transferred in to fresh microfuge tubes and equal volume of absolute alcohol was added and the tubes were incubated overnight at -20ºC. After incubation, these tubes were centrifuged at 12000 rpm for 20 min at 4ºC. The pellet was washed with 1 ml 70% ethanol and tubes were centrifuged at 12000 rpm for 10 min at RT. The supernatant was decanted and pellet was air dried. The air dried pellet was dissolved in to 100 µl of Tris-EDTA buffer (pH 8.0).
Preparation of equilibrated phenol was mentioned in **Appendix I**

3.2.2.4 Quantitative and qualitative analysis of plasmid DNA (Sambrook and Russell 2007)

a) Spectrophotometric analysis

The purity and yield of the isolated DNA was assessed by UV spectrophotometric analysis. Plasmid DNA samples were analysed 260 nm, 270 nm and 280 nm to estimate the yield and contamination of protein, RNA, phenol. The ratio of absorption maxima at 260/280 nm near 1.8 signifies that the DNA is largely free from contamination of protein and RNA.

b) Agarose gel electrophoresis

Isolated plasmid DNA was qualitatively analysed by agarose gel electrophoresis. An appropriate amount of agarose powder was weighed according to required percentage of gel and dissolved into 0.5X TAE **(Appendix III)**. Ethidium bromide (0.5 µg/ml) **(Appendix III)**

was added to the mixture. Then the agarose mixture was allowed to gel in a gel caster with comb inserted at one side of the gel to load the samples. After the semi-solidification of gel, comb was removed and the cast was transferred to the electrophoresis tank filled with 0.5XTAE buffer. The gel was submerged in the buffer. The wells of the gel were placed towards the cathode. DNA samples were prepared by mixing it with 6x gel loading dye (6:1) **(Appendix III)** and were loaded into the wells of the agarose gel along with a DNA marker. A current at 1-5 V/cm was applied for a specified time period depending on the separation to be achieved. The images of electrophoresis analysis were captured using Gel Documentation System and documented for further analysis.

3.2.2.5 Restriction endonuclease (RE) digestion

RE reaction mixture (30µl) was prepared as mentioned in **Table 3.1 and 3.2** for single and double RE digestion of DNA samples respectively. The reaction tube was given a brief spin and then incubated at 37°C for 16 h. After incubation, the tube was heated at 65°C for 5 min. The DNA of the tube was precipitated with absolute ethanol and kept at -20^{o}C for 2 h. The tube was centrifuged at 12000 rpm at 4°C for 20 min to pellet down the precipitated DNA. Supernatant was then discarded and pellet was dried in air and dissolved in 20µl of sterile Tris-EDTA solution of pH 8.0. Further, this sample was analysed on agarose gel.

Table 3.1 RE reaction mixture for single digestion

S. No.	Content	Volume/Quantity
1	Milli Q water	--µl
2	RE buffer (10X)	3µl
3	DNA	1µg/-µl
4	RE enzyme (1U/µl)	5U/-µl
5	Total	30µl

Table 3.2 RE reaction mixture for double digestion

S. No.	Content	Volume/Quantity
1	Milli Q water	--µl
2	RE (Tango) buffer (10X)	3µl (2X)
3	DNA	1µg/-µl
4	RE enzyme-1 (1U/µl)	5U/-µl
5	RE enzyme-2 (1U/µl)	5U/-µl
6	Total	30µl

3.2.2.6 Ligation

For ligation reaction total 20 µl of ligation reaction mixture containing all the components were prepared **(Table 3.3).** Vector DNA (100ng/ 5µl) and insert DNA solution were added in 1:3 and 1:6 molar ratio in 0.5 ml microfuge tube. 10X ligation buffer and calculated volume of milli Q water were added to the tube. T4 DNA ligase (1 µl; 1U/ µl) was added to the reaction mixture as mentioned in **Table 3.3.** The tube was given a brief spin and incubated at 22°C for 16 h. At the completion of 16 h, the reaction mixture was heated at 65°C for 10 min to denature the remaining enzyme. The ligation mixture was directly used for transformation.

Table 3.3 Reaction mixture for ligation

S. No.	Content	Volume/Quantity
1	Milli Q water	--µl
2	Ligase buffer (10X)	2µl
3	Vector DNA: Insert Vector	1:3 or 1:6 ratio
4	Ligase (1U/µl)	2µl
5	Total	20µl

3.2.2.7 Gel elution

Elution of the desired fragment from agarose gel was performed using gel elute^{TM} exraction kit (Sigma Aldrich, USA). The gel portion containing desired DNA fragment was excised from the agarose gel with a clean, sharp scalpel or razor blade. Excess gel was trimed to minimize the amount of agarose. The gel was weighed and sliced in a tube. Three gel volumes of the Gel Solubilization Solution were added to the gel slice and were incubated at 50-60°C for 10 min or until the gel slice was completely dissolved. To this GenElute binding column was placed into one of the provided 2 ml collection tubes. 500 mL of the Column Preparation Solution was added to each binding column and the tube was centrifuged for 1 min. Flowthrough liquid was discarded.

To this gel mixture, isopropanol (100%) of one gel volume was added and mixed. Solubilized gel solution mixture was loaded into the binding column that was assembled in a 2 ml collection tube. Tube was centrifuge for 1 min after loading the column each time. Flowthrough liquid was discarded. Wash solution (700 µl) was added to the binding column. Tube was centrifuged for 1 min. The binding column was removed from the collection tube and flow- through liquid was discarded. Binding column was placed back into the collection tube and centrifuged again for 1 min. Binding column was transferred to a fresh collection

tube. Elution Solution (50 µl) was added to the center of the membrane and incubated for 1 min. Tube was centrifuged for 1 min. Eluted DNA was stored at -20^{o}C for further use. The whole elution procedure was carried out at 12000 rpm.

3.2.2.8 Transformation of bacterial cells with plasmid DNA

a) ***Chemical method***

Transformation of *E. coli DH5α* with vector (plasmid) in the present study (Sambrook and Russell 2007; Desai 2009, Ph.D thesis) was carried out as follows:

E. coli DH5α cells were revived from glycerol stock stored at -20°C as mentioned in **Section 3.2.2.1c.** Hundred microlitre of inoculum was taken from freshly grown liquid culture and added in to fresh 10 ml sterile LB medium in a conical flask and incubated at 37°C for 2-3 hs at 200 rpm to obtain Optical density (O.D) 0.3-0.4 at 600 nm. Aseptically, the cells were transferred to 2 ml microfuge tube and kept on ice for 30 min. The cells were centrifuged at 4000 rpm for 15 min at 4^{o}C and the pellet was resuspended in 1 ml of ice cold 0.1M $CaCl_2$. The process was repeated and the cells were resuspended in 0.2 ml of ice cold 0.1 M $CaCl_2$ and stored on ice for 30 min. Subsequently plasmid DNA was added to the tube and contents of the tube were mixed gently and the tube was stored on ice for 30 min. The cells (tubes) were subjected to heat shock at 42°C for 90 sec. The tube was rapidly transferred on ice bath and incubated for 5-10 min. Then contents of the tube were transferred aseptically to 2 ml of LB medium in sterile microfuge tube. The cells were incubated at 37°C for 45 min to allow the cells to grow. After 45 min, cells were recovered by centrifugation at 6000 rpm for 5 min at 4°C. Pellet was resuspended in 200 µl sterile LB. The resuspended cells were transferred to Luria Agar (LA) plate containing the appropriate antibiotic and spreaded uniformly across the plate. Luria agar plate was as prepared as mentioned in **Section 3.2.2.1b.** Plates were incubated at 37°C for 16 h. Positive transformants or colonies appeared on plate were selected and grown in 2 ml LB medium under the above mentioned culture conditions.

b) Electroporation method

This method was used for the transformation of *Agrobacterium tumefaciens* cells with plasmid DNA.

(i) Preparation of competent cells (*Agrobacterium tumefaciens* LBA 4404) for transformation by electroporation (Sambrook and Russell, 2007; Desai 2009)

Following steps were performed under sterile conditions.

Agrobacterium tumefaciens cells were revived from glycerol stock as mentioned in **Section 3.2.2.1d.** After 20 h, 1 ml of inoculum of freshly grown culture and added in fresh 50 ml of sterile LB medium in 250 ml conical flask. Culture flasks were incubated at 28°C for 6-7 h at 200 rpm to obtain optical density 0.5-0.6 at 600 nm. Culture flasks were cooled on ice for 15 min. Culture was taken in 15 ml centrifuged tubes and centrifuged at 4000 rpm at 4^{o}C for 15 min. Supernatant was discarded and pellet was resuspended in 100 ml ice cold sterile MQ water. Cells were pelleted down by centrifugation and supernatant was discarded. Cells were washed with 10% glycerol and incubated with 10% glycerol for 1 h. Again cells were centrifuged and resuspended in 10% glycerol and incubated for 15 min. Cells were pelleted down by centrifugation and supernatant was discarded. Finally, cells were resuspended in 1 ml 10% glycerol. Aliquots of resuspended cells were prepared in 0.5 ml sterilized microfuge tubes and store at –80^{o}C.

(ii) Transformation of vector in to *Agrobacterium tumefaciens* by electroporation

Electroporation cuvette (0.1 cm) was placed in ice for 10 min. Stored competent cells at -80^{o}C were taken and thawed on ice.

Fifty microlitre of competent cells were placed in the 1.5 ml microfuge tube. 1 µl (1µg) of vector DNA was gently mixed in microfuge tube. Mixture of competent cells and DNA was placed in the internal side of the cuvette. Exterior sides of cuvette were dried with tissue paper and it was placed in electroporator apparatus. Pulse button was pressed once and waited to hear a beep for completion of process. The parameters for electroporation were set in the programme as per following specifications.

Voltage = 2.4 kV; Capacitance = 25 mF; Resistance = 200 W

Cuvette was removed from the apparatus and 1 ml of SOC medium was applied as quickly as possible **(Appendix II)**. Then all the cells with SOC medium were taken out into a new 1.5 ml microfuge tube and incubated at 28^{o}C for 2 h with 200 rpm. Cells were placed or spreaded on petriplates containing LA medium containing appropriate antibiotics (Rimfampicin and kanamycin). Plates were incubated at 28^{o}C for 48 h for development of transformed colonies.

3.2.3 Plant tissue culture work

For plant tissue culture work of the study, following methodology and protocols were used

3.2.3.1 Culture medium: *N. tabacum* plants used for the study were maintained in MS medium which was prepared with the help of various stock solutions as per the composition mentioned by Murashige and Skoog (1962).

a) ***Preparation of macronutrient stock***: Chemicals used in the preparation of macronutrients are listed in **Table 3.4.**

MS stock concentration : 10X

Total volume : 500 ml

Working Volume : 50 ml/litre

Working Concentration : 1X

Table 3.4 Preparation of stock solution for macronutrients

S. No.	Chemicals	Quantity (gm/10L)
1	KNO_3	19
2	$NH4NO_3$	16.5
3	$MgSO_4.7H_2O$	3.7
4	$CaCl_2.2H_2O$	4.4

For the preparation of macronutrient stock solution potassium nitrate and magnesium sulphate were weighed and dissolved in small amount of DDW in one flask and in second flask ammonium nitrate and calcium chloride were weighed and dissolved in DDW. Both the solutions were mixed together and final volume was made up with DDW to 500 ml using volumetric flask. The working volume of the stock solution was 50 ml/litre.

b) Preparation of micronutrients stock solution

Micronutrients stock solution was prepared using the different concentration of microelements. The microelements used in the stock solution are listed in **Table 3.5**.

MS stock concentration : 10X

Total volume : 400 ml

Working Volume : 40 ml/litre

Working Concentration : 1X

Table 3.5 Preparation of stock solution for micronutrients

S. No.	Chemicals	Amount (mg/10L)	Stock Solution	Volume to be used (ml)
1	$MnSO_4.4H_2O$	169	200 mg/ 50 ml	42.25
2	H_3BO_3	62	100 mg/ 50 ml	31
3	$ZnSO_4.7H_2O$	86	100 mg/ 50 ml	43
4	KI	8.3	10 mg/ 25 ml	20.7
5	$NaMoO_4$	2.5	10 mg/ 25 ml	6.25
6	$CoCl_2.6H_2O$	0.25	10 mg/ 25 ml	0.625
7	$CuSO_4.5H_2O$	0.25	10 mg/ 25 ml	0.625

The concentration of microelements in the required medium is very less; hence, stock solution of the individual salt was prepared and stored at 4-8 ºC in a glass bottle. The required quantity of the each salt solution was taken and final volume was made up to 400 ml with DDW and stored in refrigerator. The working concentration of stock solution was 40 ml/litre.

c) Preparation of iron salt stock solution

Iron salt stock was prepared as mentioned in **Table 3.6.**

MS stock concentration : 10X

Total volume : 200 ml

Working Volume : 20 ml/litre

Working Concentration : 1X

Table 3.6 Preparation of iron salt stock solution

S. No.	Chemicals	Quantity (mg/10L)
1	$FeSO_4.7H_2O$	278
2	$Na_2EDTA.2H_2O$	373

Both the salts were weighed and dissolved in two separate flasks. Both the flasks were heated to the boiling and mixed together. Again mixture was boiled for one more min and allowed it to cool down to the room temperature and final volume of the mixture was adjusted up to 200 ml. Stock solution was stored in the amber colour bottle.

d) Stock solution preparation for vitamins

Stock solution of each vitamin were separately prepared in DDW and stored in glass bottles at 4-8ºC.

Table 3.7 Stock solution of vitamins

S. No.	Vitamins	Quantity (mg/l)	Stock Solution 25 ml	Volume to be used (ml/l)
1	Thiamine	0.1	10 mg	0.25
2	Pyridoxine HCl	0.5	10 mg	1.25
3	Nicotinic acid	0.5	10 mg	1.25
4	Glycine	2.0	100 mg	0.5

e) Stock solution preparation of plant growth regulators

Plant growth regulators such as auxins and cytokinin were initially dissolved in very small amount of 0.1N NaOH and 0.1N HCl solution respectively and final volume was made up with milli Q water.

(i) Preparation of 1mM 6-Benzyladenine (BA) stock solution

5.6 mg of BA was weighed and dissolved in to small volume of 0.1N HCl and final volume was made up to 25 ml with DDW.

(ii) Preparation of 1mM 1- Naphthylacetic acid (NAA) stock solution

4.66 mg of NAA was weighed and dissolved in to small volume of 0.1N NaOH and final volume was made up to 25 ml with DDW.

(iii) Preparation of 1mM kinetin (kn) stock solution

5.4 mg was weighed and dissolved in to small volume of 0.1N HCl and final volume was made up to 25 ml with DDW.

(iv) Preparation of 1mM Indole-3-acetic acid (IAA) stock solution

4.4 mg was weighed and dissolved in to small volume of 0.1N NaOH and final volume was made up to 25 ml with DDW.

(v) Preparation of Folic acid stock solution

25 mg was weighed and dissolved in to small amount of water. Final volume was made up to 25 ml with DDW.

Storage: Growth regulators solutions were stored at 4 to 8 ºC for 1 month.

3.2.3.2 MS medium preparation

Sucrose, KH_2PO4 and mesoinositol were weighed according to medium composition and experimental design. All components were dissolved in small amount of distilled water. To this required volumes of solutions of macrosalts, microsalts, iron salts, vitamins and plant growth regulators were added from respective stock solutions. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or 0.1 N HCl using pH meter. The final volume of the medium was adjusted with distilled water. The medium was equally dispensed in the flask containing agar (as per the requirement of medium per flask). The culture flasks were plugged with the cotton plug and covered with butter paper to prevent the water entrapment. Medium was autoclaved at 121ºC and 15 lbs for 15 min.

a) Preparation of MS Medium for maintenance and rooting of N. tabacum culture

Maintenance of *N. tabacum* culture was done by the excision of nodal segment of the *in vitro* grown shoot culture. The MS medium prepared for the maintenance of *N. tabacum* contained the 0.9% agar. The composition of the maintenance medium is mentioned in **Table 3.8.**

Table 3.8 Composition of MS medium for maintenance of *N. tabacum*

S. No.	Medium Components	Quantity/ Volume
1	MS-A	50 ml
2	MS-B	40 ml
3	MS-F	20 ml
4	Thiamine HCl	1 ml
5	Sucrose	30 gm
6	KH2PO4	170 mg
7	Meso-Inositol	100 mg
8	Combination of 2.263 µM Folicacid+13.95µM kinetin+5.71µM IAA	1 ml

b) Preparation of MS Medium for shoot regeneration of N. tabacum from leaf discs

This medium was used for the regeneration of multiple shoots from the leaf explants or discs of *N. tabacum*. This medium contained the 0.8 % of agar. The composition of the regeneration medium is mentioned in **Table 3.9.**

Table 3.9 Composition of MS medium for multiple shoot regeneration

S. No.	Medium Components	Quantity/ Volume
1	MS-A	50 ml
2	MS-B	40 ml
3	MS-F	20 ml
4	Thiamine HCl	1 ml
5	Sucrose	30 gm
6	KH2PO4	170 mg
7	Meso-Inositol	100 mg
8	BA	4.4 ml
9	NAA	0.53 ml

3.2.3.3 Culture conditions

The inoculated culture flasks were placed in culture room with 16:8 h (light:dark) photoperiod with cool white fluorescent lights (Philips, India with total light intensity of 3.5 X 10^{-17} μmoles/m^2/s), at 24± 2^oC temperature and 70-80 % humidity.

3.2.3.4 Observation

Observations were taken on daily/weekly/monthly basis as per experimental design. Photographs of different stages of growth of *in vitro* cultures were taken.

3.2.3.5 Maintenance of *in vitro* culture of *N. tabacum*

Excised nodal segment of *N. tabacum* was used for the maintenance of *in vitro* culture of *N. tabacum*. Excised explants were inoculated on MS medium which was as prepared as mentioned in **Section 3.2.3.2b** and components were added as mentioned in **Table 3.8.** Cultures were incubated under suitable culture conditions as mentioned in **Section 3.2.3.3**.

3.2.3.6 Plant genetic engineering work

a) Basta sensitivity test

Sensitivity of leaf explants of *Nicotiana tabacum* was analysed on Basta®. The concentration of Basta® ranging from 0.25µg/ml to 6.0µg/ml was used for test. Basta® was added to the medium after sterilization of medium under aseptic condition.

b) Agrobacterium-mediated genetic transformation of Nicotiana tabacum

- **Preparation of MS medium**

Preconditioning medium: MS medium for multiple shoot regeneration from leaf disc was prepared **(Section 3.2.3.2b and Table 3.9).** 30 ml of medium was distributed in each flask containing 0.8% agar and sterilized by autoclaving, this medium was used for the preconditioning of the leaf explants.

Cefotaxime containing medium: After co-cultivation, to prevent the excessive growth of the *Agrobacterium*, cefotaxime antibiotic (500µg/ml) was added in the MS medium after sterilization.

Selection medium: Selection of the putative transformants was done on MS medium containing cefotaxime (500µg/ml) and basta® (1µg/ml) antibiotics.

- **Inoculation and pre-conditioning of leaf explants**

All the steps of inoculation were carried out under aseptic conditions in laminar air flow hood. The leaves from *in vitro* grown plantlets of *N. tabacum* were used as explants. Leaf discs were prepared using borer and inoculated on previously sterilized MS medium and incubated for 48 h. Inoculated cultures flasks were placed in a culture room with 16:8 h (light:dark) photoperiod with cool white fluorescent lights (light intensity of 3.5 X 10^{-17} µmoles/m^2/s) with 24 ± 2°C temperature and 70-80 % humidity.

- **Growth of *A. tumefaciens* LBA4404 cultures (harbouring vectors)**

Inoculum (100 µl) of *A. tumefaciens* culture (pregrown or maintenance culture) was inoculated in 10 ml LB medium containing rifampicin (50 µg/ml) and kanamycin (50 µg/ml). The culture was incubated for 20 h at 28°C at 200 rpm in orbital shaker. After 20 h, 1 ml of freshly grown culture was inoculated in 50 ml (2 flasks) LB medium containing rifampicin and kanamycin in appropriate concentration and incubated the culture for 6-7 h at 28°C at 200

rpm in orbital shaker till absorbance reaches 0.5 - 0.6. This *Agrobacterium* culture was used for transformation studies.

- **Infection of pre-conditioned leaf explants with *Agrobacterium* culture**

A. tumefaciens (OD_{600} = 0.5-0.6) culture was used for infection of pre-conditioned leaf explants. Cultures were taken in 15 ml centrifuge tubes and centrifuged at 4000 rpm for 15 min at 4^oC. Supernatant was discarded and pellet was resuspended in 30 ml liquid MS medium. 6 ml of culture was taken in petriplates and explants were transferred to the petriplates and co-cultivated with *Agrobacterium* culture for 2 min. Co-cultivated explants were again inoculated on the same pre-conditioning medium. Cultures were incubated for 48 h at optimum culture conditions in culture room.

- **Transfer of infected leaf explants on cefotaxime containing medium**

After 2 days of co-cultivation, treated explants were transferred on MS medium containing cefotaxime antibiotic. Cultures were incubated for 12 days in culture room.

- **Subculture of infected leaf explants on selection medium**

After 12 days, all the infected explants were transferred to the selection medium containing appropriate antibiotics for the selection of putative transformants. After the interval of every 28 days, putative transformants were subcultured on the selection medium of same composition for the proper growth and maintenance of transformants.

c) Rooting of transformed plants

Elongated shoots were excised from *in vitro* grown culture and subjected to the rooting MS medium (**Section 3.2.3.3a and Table 3.9).** Cultures were incubated under suitable culture conditions as mentioned in **Section 3.2.3.3.**

d) Hardening of transgenic plants

Rooted transgenic plants were transferred in to pot containing mixture of soil and sand (1:1) for hardening in *in vivo* conditions. Plants were nourished every alternate day with sterile water and liquid basal MS medium (50:50).

e) Analysis of transgenic plants

Transgenic plants were analysed to confirm the integration of erythropoietin transgene in to the genomic DNA of *N. tabacum* and expression of erythropoietin was analysed at mRNA

level and at protein level. HPLC analysis was also performed to detect the level of nicotine in nontransformed and transformed plants.

3.2.4. Analysis of transformed bacteria and transgenic plants

Analysis of transformed bacterial cells was performed using colony PCR method. Transformed plants were analysed for the confirmation of the transgene integration and its expression in *N. tabacum.*

3.2.4.1 Colony PCR screening for confirmation of transformed bacterial cells

PCR reaction mixture of 50µl containing; Forward primer 10µM (100µM), reverse primer 10µM (100µM), 1X taq buffer (10 X), 1.5mM $MgCl_2$ (25 mM), 0.2 mM dNTPs (10 mM), Taq polymerase (1U/1µl) and template DNA (100-200ng) or transformed single colony was prepared. Final volume of the reaction mixture up to 50µl was made up with milli Q water. PCR conditions were set for the amplification of specific transgene (as mentioned in Table 3.10).

Table 3.10 PCR conditions for amplification of cEPO and Syn7

PCR of cEPO			
S. No.	**Step**	**Temperature ^{o}C**	**Minute**
1	Initial Denaturation	94	7
2	Denaturation	94	1
3	Annaeling	58.5	1
4	Extension	72	2
In above programme step 2-4 were repeated 30 times			
5	Final Extension	72	7
PCR of Syn7			
S. No.	**Step**	**Temperature ^{o}C**	**Minute**
1	Initial Denaturation	94	7
2	Denaturation	94	1
3	Annaeling	58.5	1
4	Extension	72	2
In above programme step 2-4 were repeated 30 times			
5	Final Extension	72	7

3.2.4.2 Isolation of total genomic DNA of *N. tabacum*

Genomic DNA was isolated from the non-transformed and transformed plants of *N. tabacum* using Cetyl triethyl ammonium bromide (CTAB) method (Stewart and Via 1993; Stewart 1997).

100 mg of fresh leaves were crushed with 1 ml of CTAB buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-Cl, 20 mM EDTA, 2% PVP, 5 mM ascorbic acid and 6 µl/ ml *β*-mercaptoethanol) and then transferred to 2 ml eppendorf tube. The tube was incubated at 65°C for 30 min. To each tube, 1 ml of chloroform: isoamyl alcohol (24:1) was added and mixed properly. The tube was centrifuged at 3000 g for 5 min at 24°C. The supernatant was transferred to fresh tube and 0.7 volume of isopropyl alcohol was added to it. The tube was incubated for 60 min at -20°C for precipitation and centrifuged at 6000 g for 10 min at 24°C to pellet down the precipitated genomic DNA. The supernatant was discarded and pellet was washed with 70% alcohol. The tube was again centrifuged at 6000 g for 10 min at 24°C and the pellet was air dried. The pellet was finally dissolved in 50 µl of TE buffer, pH 8.0 with 2 µl/ml of RNase and incubated overnight at 37°C in waterbath. The isolated genomic DNA was then analyzed using agarose gel electrophoresis and spectrophotometric quantification.

3.2.4.3 PCR confirmation of putative transformants

Multiplex PCR was performed for the detection of integrated transgene in to genomic DNA of *N. tabacum.*

The volume of reaction mixture was set up to 50 µl containing 10 pmol of each primer (Forward primer and Reverse primer), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1X Taq buffer 1X, 2U (unit) of Taq polymerase and 50-100 ng of template. The prepared reaction mixture containing tubes were subjected to PCR and PCR steps were as followed as mentioned in **Table 3.11**. The PCR products were analysed on 2% agarose gel containing 0.5 mg/ml ethidium bromide and visualized under UV- transilluminator and photographed using gel documentation system (Bio-Rad, USA). GeneRuler 50 bp DNA ladder (Fermentas, USA) was used as a marker to compare the size of amplified product.

Table: 3.11 Multiplex PCR conditions for the amplification of *Bar*, Syn7, EPO and *RP L25* genes.

S. No.	Step	Temperature ^{o}C	Minute
1	Initial Denaturation	94	7
2	Denaturation	94	1
3	Annaeling	58.5	1
4	Extension	72	2
In above programme step 2-4 were repeated 30 times			
5	Final Extension	72	7

3.2.4.4 Southern blot analysis of transgene integration

Southern blot analysis was performed to confirm the stable integration of transgene in the genomic DNA of transgenic plants using DIG DNA Labeling and Detection Kit - Roche Life Science.

This analysis was performed in four major steps

STEP 1

i) Restriction Enzyme Digestion

Genomic DNA of transformed and non-transformed plants was subjected to *EcoR*I RE enzyme to digest the genomic DNA in to small fragments which was observed on 1% agarose gel.

ii) DNA Denaturation

After electrophoresis, DNA present in gel was denatured by soaking the gel in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 45 min. Gel was rinsed with water for 10 min.

iii) DNA Neutralisation

After the rinse with water, gel was placed in neutralising solution (1.5 M NaCl, 1M Tris-HCl, pH7.4) for 30 min.

STEP 2

Blot Transfer

- This step was performed to transfer the whole DNA present in agarose gel to the membrane using capillary blot transfer method for hybridization.
- The PVDF membrane was immersed in hybridization solution (5xSSPE, 0.02% SDS and final volume of solution was adjusted with H_2O) and incubated for at least 1 h at 62^{o}C.

STEP 3

Hybridization of membrane with probe

- After the incubation, DIG labeled probe (Given in the kit) was denatured by first adding H_2O up to a final volume of 100 µl, and then boiled for 10 min in a screw-top microfuge tube, then allowed them to cool rapidly on ice. Denatured probe was added to the hybridization solution in the bag and mixed well. Blot was incubated with this mixture in bag overnight at 62^oC for hybridization.
- After the hybridization, membrane was taken out from the bag and washed twice for 5 min each, at room temperature with 2x SSPE + 0.1% (w/v) SDS (the "low stringency" wash), and twice for 15 min each at 62^oC for 15 min with 0.1 x SSC + 0.1% (w/v) SDS (the "high stringency" wash).

  Note: Hybridization solution contained 5x SSPE + 0.1% (w/v) N-Iaurylsarcosine; 0.02% (w/v) SDS + 2% (w/v) blocking reagent (provided in kit). The solution was prepared about 1 h before use by heating to 55 to 70^oC and then stirred to dissolve the blocking reagent. The hybridization and wash solutions were prepared from stock solutions of 20 x SSC, 10% (w/v) N-laurylsarcosine and/ or 10% (w/v) SDS.

  Note: SSPE buffer preparation was mentioned in ***Appendix V***

STEP 4

Colour Development

- Membrane was dried after hybridization and washed briefly in buffer 1.
- Membrane was incubated in 100 ml buffer 2 for 30 min.
- Membrane was rinsed in buffer 1, and then incubated for 30 min in 10 ml antibody solution (2 µl in 10 ml). Membrane was kept with antibody in shaker for constant shaking (shaking is crucial at this stage). The antibody antidigoxigenin-antibody conjugated to alkaline phosphatise solution (provided in kit) was diluted in 1:5000 dilution ratio in buffer 1.
- Membrane was washed twice for 10 min each with 100 ml of buffer 1.
- Membrane was equilibrated for 2 min with up to 100 ml of buffer 3 either in a Petri dish, or sealed plastic bag, with 10 ml of colour solution. Left the membrane in the dark for up to 3 hs. Avoid shaking or mixing during the colour development stage.

- Membrane was rinsed in H_2O and allowed to dry at 37^oC and stored at room temperature, colour fades if exposed to light for long period of time.

Note: Procedures of step 1 and 2 were referred from (Sambrook and Russell 2007) and procedures of step 3 and 4 were referred from kit protocol. All the buffer preparation of colour development of membrane was mentioned in Appendix V.

3.2.4.5 HPLC analysis of Nicotine in *N. tabacum* (transformed and non-transformed plants)

HPLC analysis was performed to analyse the nicotine content in non-transformed and transformed plants of *N. tabacum.*

a) ***HPLC Method validation***

It was performed according to International conference on Harmonisation (ICH) guidelines (Website:http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf)

For the method validation, Limit of Detection (LOD) and Limit of Quantification (LOQ) were indentified. LOD is the lowest amount of analyte in a sample which can be detected but cannot be quantified. The LOD was estimated by the standards (n=6) signal-to-noise ratio 3:1. Limit of Quantification (LOQ) is the lowest amount of analyte in a sample which can be quantified. The LOQ of standards (n=6) was estimated with signal-to-noise ratio 10:1. Precision was analysed by the intraday and inter day precision.

b) ***Method validation for Nicotine***

Stock solution of 100 ppm of nicotine standard was prepared with methanol (HPLC grade). Different concentration such as 1, 5, 10, 20, 50, 100 ppm of nicotine standard was prepared from the stock solution of the nicotine. 500µl of each concentration was taken in glass sample vial. Each sample was injected in triplicates. Intermediate lower (0.3 ppm), medium (8 ppm) and higher concentration (80 ppm) were also taken for precision analysis and injected in triplicates. HPLC chromatograms were observed and area under curve was calculated for each concentration.

c) *Sample preparation*

Fresh Leaves of transformed and non-transformed plants were dried in hot air oven at 45^{o}C for 24 h. Dried leaves were crushed in to fine powder using pestle-mortar. 100 mg of the dried leaf powder was taken in 15 ml fresh centrifuge tube. 10 ml of 40% methanolic sodium phosphate buffer pH 7.0 was added to the tube and mixed properly by vortex. Then it was kept in shaker at 150 rpm overnight at 28^{o}C (Rex et al. 2014). At the next day, extract was filtered using syringe filter with Millipore filter (0.22µm). 10 µl of the extract was injected in to the column.

3.2.4.6 Plant RNA isolation and integrity analysis

In this section, protocols regarding the isolation of RNA, its integrity analysis and expression of mRNA of erythropoietin are described.

a) RNA isolation

Total RNA was isolated from fresh leaves of transformed and non-transformed plants of the *N. tabacum*. The protocol was followed as per the instructions.

Leaf plant material (100 mg) was taken and crushed using a pestle and mortar with 450 µl of RLC buffer. Crushed sample was transferred in to 2 ml microfuge tubes and tube was vortexed vigorously. Lysate was transferred in to QIAshredder spin column placed in a 2 ml collection tube, and tube was centrifuged for 2 min at 12000 rpm. The supernatant of flow-through was transferred to new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. 0.5 volume of ethanol (96-100%) was added to the cleared lysate, and mixed immediately by pipetting. The sample (usually 650 µl) was transferred including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Tubes were centrifuged for 15 sec at 10000 rpm. The flow-through was discarded. 700 µl of buffer RW1 was added to the RNeasy spin column and centrifuged for 15 sec at 10000 rpm to wash the spin column membrane. Flow-through was discarded. 500 µl of buffer RPE was added to the RNeasy spin column and centrifuged for 15 sec at 10000 rpm to wash the spin column membrane. Flow-through was discarded. 500 µl of buffer RPE was added to the RNeasy spin column and centrifuged for 2 min at 10000 rpm to wash the spin column membrane. RNeasy spin column was placed in new 2 ml collection tube and centrifuged at 12000 rpm for 1 min. RNeasy spin column was placed in a new 1.5 ml collection tube and

30µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at 10000 rpm to elute RNA. RNA elution step was repeated again using another 30–50 µl RNase free water.

b) Formaldehyde agarose (FA) gel electrophoresis

Formaldehyde agarose gel electrophoresis is used to check the size and integrity of RNA preparations. RNA can form many different secondary or tertiary structures which affect its separation in an electrical field. Formaldehyde is used to keep the RNA denatured. After electrophoresis, the RNA was observed under gel documentation unit.

Formaldehyde agarose gel preparation

1.5 g agarose was weighed and 10 ml of 10X FA gel buffer was added and volume was made up to 100 ml with RNase free water. This mixture was heated to melt agarose and cooled to 65^{o}C in water bath. 1.8 ml of 37% (12.3M) formaldehyde and 1µl of 10mg/ml ethidium bromide stock solution was added to gel mixture. Mixed thoroughly and poured on to gel caster and allowed to semi solidify. Gel was equilibrated in 1X FA running buffer for 30 min.

RNA sample preparation for formaldehyde agarose gel electrophoresis

1 volume of 5x RNA loading buffer was added to 4 volumes of RNA sample (e.g. 10 µl of loading buffer and 40 µl of RNA) and mixed. Samples were incubated for 3–5 min at 65°C and chilled on ice. Then samples were loaded on equilibrated FA gel.

Gel running conditions

Gel was run at 5–7 V/cm in 1x FA gel running buffer.

Note: *Preparation of 10x FA gel buffer, 1x FA gel running buffer, 5x RNA loading buffer mentioned in Appendix IV.*

3.2.4.7 Reverse transcription for cDNA preparation

Total isolated RNA was subjected to cDNA preparation using Iscript^{TM} (Biorad) reverse transcription supermix. Protocol was followed as mentioned in the kit.

Reaction mixture of 20 µl was prepared containing 200 ng of RNA and 5X Iscript reverse transcription supermix solution. Final volume of reaction mixture was made up to 20 µl with milli Q water. Reaction mixture was kept in PCR under following programme and conditions.

Step 1: 25^{o}C for 10 min

Step 2: 42^{o}C for 35 min

Step 3: 85^{o}C for 5 min

Reaction mixture tubes were taken out from the PCR and stored at -20^{o}C for further use in semiquantitative PCR and quantitative real time PCR analysis.

3.2.4.8 Qualitative analysis of mRNA expression of EPO

Prepared cDNA (from total RNA) was subjected to PCR analysis using transgene specific primers. PCR conditions were followed as mentioned in **Section 3.2.2.9.** This experiment was performed to analyse the concentration of mRNA expressed in non-transformed and transformed plant samples which was observed and analysed on agarose gel after PCR amplification.

3.2.4.9 Quantitative mRNA expression analysis of EPO using Real Time PCR

Real time PCR experiment was perfomed according to MIQE guidelines (Bustin et al. 2009)

Real-time PCR was carried out using Power SYBR Green PCR Master Mix (Applied BioSystems) with step one real time PCR instrument (ABI) for the quantitative mRNA expression analysis of cEPO and endogenous control gene (ribosomal protein (RP) L25). This method is cost effective and it monitors the amplification of a target sequence in real-time using fluorescent technology.

a) Primer designing

The primer sequences were designed to target a specific sequence in the region of the cDNA of EPO and endogenous control gene ribosomal L25 gene of the expressed mRNA. The designing of primers was performed by Primer Express Software V 3.0 (Applied Biosystems). Software finds a sequence that is specific for the desired target. Designed primer sequences for EPO and ribosomal L25 gene were aligned using BLASTn tool (NCBI) with EPO gene and *N. tabacum* genome to analyse the specificity of the primer and to assure that no homology sequence exist in the same gene or genome.

For the SYBR green strategy the amplicon size should be 50-150 bp. The melting temperature (Tm) for the primers should be 58-60°C and GC contents should be in the range of 30-80%) (Guidelines from Applied biosystems).

b) PCR efficiency

The efficiency of PCR needs to be checked on the basis of Ct value. A dilution series amplified under low efficiency conditions could yield a standard curve with a different slope from one amplified under high efficiency conditions. The PCR efficiency is dependent on the assay, the master mix performance and sample quality. The PCR efficiency between 90 and 110% is considered as the acceptable range. PCR efficiency (E) was calculated by preparing a dilution series of cDNA of target (EPO) and endogenous control genes (RP L25). Based on the dilution series, a standard curve was generated and PCR efficiency was calculated using the formula: $E = (10^{-1/slope} - 1) \times 100$; where slope refers to the slope of the standard curve. In a 100 % efficient PCR, the DNA copies will increase by 10-fold in 3.33 cycles.

c) Preparation of reaction mixture

Reaction mixture of 10µl/well was prepared containing 100 ng of cDNA (arbitrary), 300 nM of each primer and 1x SYBR green. Final volume of the reaction mixture was made up to 15µl with milli Q water. Reaction mixture was transferred in wells of real time PCR plate. Plate was centrifuged at 2000 rpm for 5 min. PCR reaction was run to determine Ct values.

For the PCR efficiency, different dilutions of cDNA were prepared and Ct values were analysed.

d) Real time PCR conditions and cycles

Step 1	95 ^{o}C	10 min
Step 2	95 ^{o}C	15 sec
Step 3	60 ^{o}C	1 min
Step 4	72 ^{o}C	40 sec
Melt Curve		
Step 5	95 ^{o}C	15 sec
Step 6	60 ^{o}C	1 min
Step 7	95 ^{o}C	15 sec

3.2.4.10 ELISA assay for the detection and quantification of erythropoietin protein in putative transformants

Indirect Elisa was performed to detect and quantify the erythropoietin protein in the plant extract of N. tabacum.

a) Total protein extraction from plant leaves

Total protein was extracted from *N. tabacum* (Park et al. 2007).

500 mg of fresh plant tissues was grinded with 2 ml of sodium phosphate buffer (50mM $NaPO_4$, pH 7.8; 5mM EDTA; 0.05% beta- mercaptoethano; 1mM Phenylmethylsulphonyl fluoride) in pestle- mortar. Homogenate was taken in a tube and placed on ice. Samples were centrifuged at 12000g for 15 min at 4^oC. Supernatant was transferred to the new microfuge tube and stored at -20^oC for Elisa assay.

b) Total protein estimation using Bradford method

Total extracted protein from transformed and non transformed plants of *N. tabacum* was performed using Bradford assay (Bradford 1976).

Coomassie Brilliant Blue G-250 0.01% was dissolved in 4.7% (of 95%) ethanol. To this solution 8.5% (of 85% w/v) phosphoric acid was added and solution was diluted up to a final volume of 100 ml. Bovine serum albumin (BSA) was added in the test tube ranging from 2 µl to 12 µl and its volume was made up to 200 µl with milli Q water. Bradford reagent (1ml) was added in each tube. In blank tube BSA was not added. Samples were mixed properly using vortex. Absorbance was measured at 595 nm (after 2 min and before 1 h) against a blank. The concentration of known protein was plotted against the corresponding absorbance for standard curve preparation. Prepared standard curve was used to determine the concentration of protein in unknown samples (by comparison with the known concentration of proteins mentioned in standard curve).

c) ELISA Assay

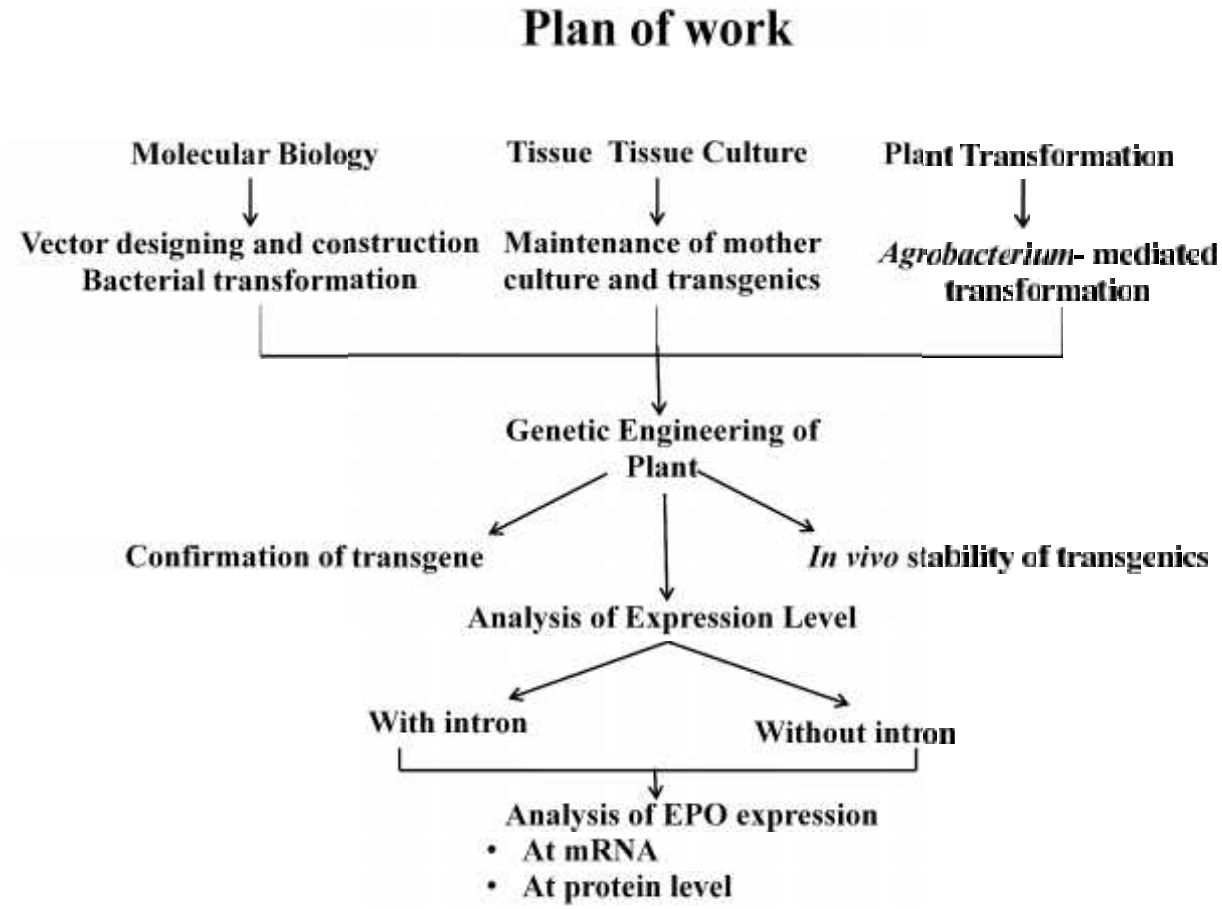
Protein extract and standard were diluted in coating buffer or carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$; pH 9.6). Each concentration of protein was coated in 5 replicates in wells. Coated plate was covered properly with aluminium foil and incubated at 4^oC overnight. Plate was washed thrice with 1X PBS (10mM Na_2HPO_4, 3mM KH_2PO_4, 100mM NaCl, 3mM KCl, 0.05 % tween-20; pH7.4). Blocking buffer (5% casein hydrolysate in 1X PBS buffer)

200 µl was added in the coated wells of plate. The plate was covered with aluminium foil and incubated for at least 2 h at room temperature and washed thrice with 1X PBS. Primary antibody of 1:5000 dilutions (prepared in 5% blocking buffer) was prepared and dispensed in each well. The plate was covered with aluminium foil and incubated for at least 2 h at room temperature. Plate was washed three times with 1X PBS. Secondary antibody of 1:7000 dilutions (prepared in 5% blocking buffer) was prepared and 100 µl dispensed in each well. Plate was incubated for 2 h and washed with PBS buffer thrice. 100 µl of TMB substrate solution was dispensed per well with a multichannel pipette. After 30 min, 100 µl of stop solution was added (0.2 M H_2SO_4) to the wells. The absorbance (optical density) was measured under plate reader at 450 nm. For control, some wells of the ELISA plate were coated with 100 µl of coating buffer and these wells were treated as same as the other sample wells of the plate. Standard curve was prepared with the different concentration of the erythropoietin. Concentration of unknown protein was calculated by the standard curve of erythropoietin. For the normalisation, absorbance of the control samples were subtracted with the absorbance of the test samples.

Chapter 4

Results and Discussions

This chapter of the thesis deals with the detailed results obtained in various experiments carried out to establish the proof of our concept and to prove the hypothesis.



The execution of the study was planned focusing on three major parts i.e. molecular biology, plant tissue culture and genetic transformation of the plants. Bacterial transformation and vector construction were performed under molecular biology section. Two vectors namely **test expression** vector containing cDNA of erythropoietin along with intron Syn7 and **control expression** vector containing only cDNA of erythropoietin (without intron Syn7) have been constructed. Maintenance of *in vitro* culture was carried out under the plant tissue culture part. Plants were transformed using the *Agrobacterium*-mediated genetic

transformation method. Presence of transgenes was confirmed in transgenic plants using PCR and southern blot techniques. *In vivo* stability of transgenic plants were analysed by the hardening of plants. Expression of erythropoietin in transgenic plants at mRNA level and protein level was confirmed by the real time PCR and ELISA assay respectively.

4.1 Molecular biology

Advances in the molecular biology techniques have provided a way to construct the desired vector with the desired transgene using restriction digestion and ligation techniques. Three plasmid vectors were used for the present study.

4.1.1 Vectors details

4.1.1.1 Vector pFGC5941- This plasmid vector pFGC5941 was selected to be used as expression vector for the present study because this vector has various multiple cloning (RE) sites which were unique and not present in the other inserts of the study such as in EPO and Syn7 sequences at any random site. This vector has tobacco mosaic virus omega leader sequence which makes it compatible for the enhanced translation or overexpression of the protein. It is a 11.4 kb plasmid vector having T-DNA region present between the left border and right border sequences. T- DNA region of the vector consists following regions (**Figure 4.1**).

i. Kanamycin resistance gene present as a selectable marker for bacterial cells.

ii. *Bar* gene as a selectable marker under the control of mannopine synthase 2' (MAS2') upstream and downstream region. *Bar* gene confers resistance against Basta®.

iii. Strong CaMV 35S promoter which is present upstream of chalcone synthase (CHSA) intron and it has octopine synthase at 3' end of the intron.

iv. There are two multiple cloning sites present at the 5' end and 3' end of CHSA intron as insertion sites for target gene sequences.

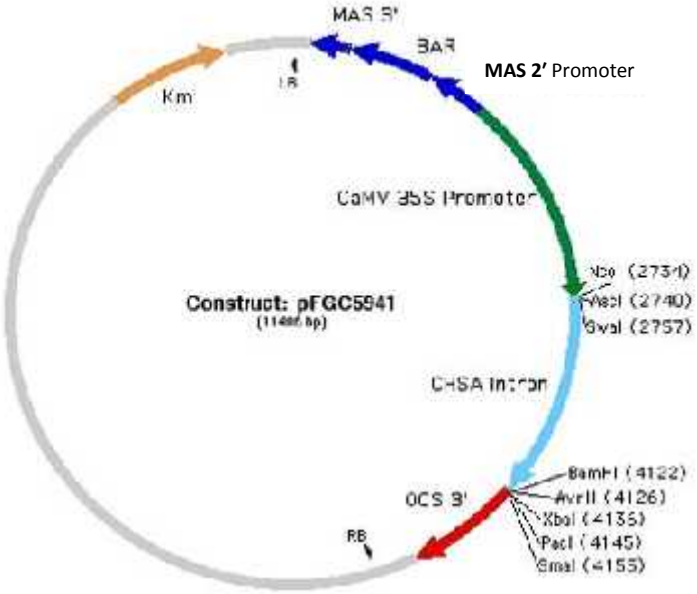


Figure 4.1: Vector map of pFGC5941

4.1.1.2 Vector pPERB33cEPO- This vector was constructed in our laboratory by a senior researcher (Dr. Priti Desai, B. V. Patel PERD Centre, Ahmedabad,Gujarat) **(Figure 4.2).** This vector is used for extracting cDNA of erythropoietin (596 bp) which has restriction enzyme site *Nco*I at the 5′ end and *BstE*II at the 3′ end of it.

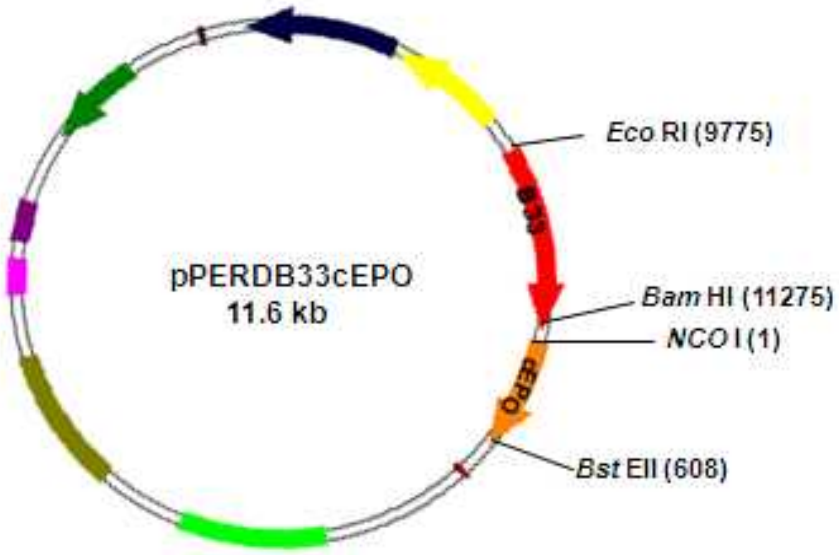


Figure 4.2: Vector map of pPERDB33cEPO

4.1.1.3 Vector pUC57Syn7- This was a shuttle vector carrying synthesized intron Syn7 sequence which was designed as per the requirement of the study. As the vector carrying intron Syn7 sequence, it was named as pUC57Syn7 in our laboratory and this nomenclature will be used hereafter for this vector **(Figure 4.3)**. It is 2.9 kb vector having backbone pUC57 vector (2.7 kb) and Syn7 sequence (246 bp). It also has various compatible RE sites around intron Syn7 sequence.

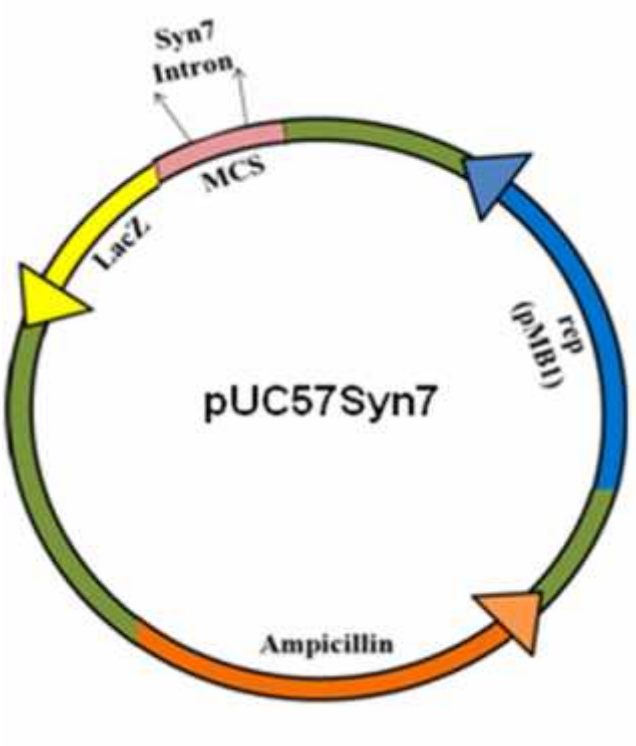


Figure 4.3: Vector map of pUC57Syn7

All these plasmid vectors were amplified in *E. coli* DH5α bacterial strain to get sufficient quantity for experimental work. *E. coli* DH5α was transformed **(Section 3.2.2.8a)** with these plasmid vectors individually for the purpose. These transformed *E. coli* DH5α Strain carrying different plasmid vectors were used in various experiments in the study.

4.1.2 *In silico* work

In silico work involves the use of bioinformatics based softwares and data banks for the analysis of nucleotide sequence of vector and trasgenes. These softwares were used for the primer designing also as mentioned in **Section 3.2.1**. NEB cutter software (New England Biolab) was used to check the accuracy of these designed sequences as well as to identify the restriction endonuclease (RE) sites present in the vector which were important for the construction of the desired vectors. Primer3 and OligoEvaluator^{TM} (Sigma-Aldrich) softwares were used to design the primers for the amplification of transgene and to analyse the melting temperature (Tm), % GC, size, and dimer in designed primer sequences. FASTA and BLAST features available in NCBI data bank were used to check accuracy of constructed vectors.

4.1.2.1 Selection, designing and procurement of intron

Intron 'syn7' has been used for the proposed study which is an already reported synthetic intron of 246 bp length. It has AT-rich nucleotides which make it compatible for proper processing by spliceosomal machinery in dicot plants. Proper processing of intron is linked with the enhanced expression of the transgene. Intron 'syn7' was selected for the study on the basis of previous research reports (Goodall and filipowicz 1989, Goodall and filipowicz 1991). For our study this intron was custom synthesized with insertion of various customized RE sites required for insertion of intron in the expression vector. RE sites for insertion of cDNA of erythropoietin with intron were also included during designing of intron Syn7 as position of the intron was decided between the promoter and coding region. Accuracy of the designed RE sites in the designed intron were checked using New England Biolabs (NEB) cutter software.

Figure 4.4 shows the sequence of intron Syn7 with all the necessary restriction sites such as *Nco*I and *BstE*II for the insertion of cEPO adjacent to the intron sequence syn7. *Asc*I and *Avr*II restriction sites for the removal of whole cassette (intron and EPO segments) from the vector. *Hpa*I restriction site was present at both 5′ and 3′ end of the intron sequence for the complete removal of intron syn7 as it is required for construction of control expression vector (intron less vector construct).

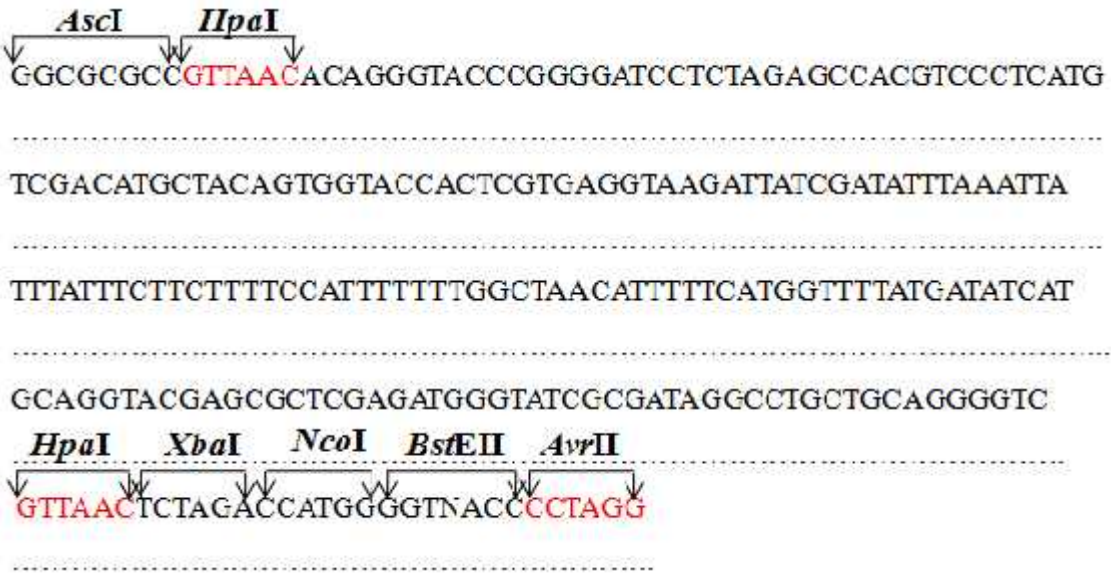


Figure 4.4: Sequence of intron Syn 7 with required RE sites

Designed intron Syn7 was sent for custom synthesis to GenScript, A USA based company provides services of gene synthesis. Custom synthesized intron sequence was sent to us by Genscript in to the cloning vector pUC57, which was named '**pUC57Syn7 insert vector'** in our laboratory (**Figure 4.5)**, as it is having our Syn7 insert.

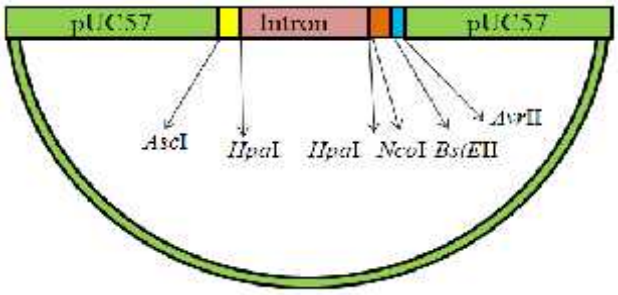


Figure 4.5: Schematic representation of pUC57Syn7 with RE sites

This vector was obtained as lyophilized powder, which was dissolved in the fresh and sterilized tris-EDTA buffer and was used for the bacterial transformation.

4.1.3 Vector construction strategy

Folllowing section of the thesis details the construction of test expression vector pFGC5941Syn7cEPO and control expression vector pFGC5941cEPO. The vector construction was carried out in two major steps described as strategy 1 and strategy 2 in the following section.Various factorial experiments were performed for excision and insertion of required fragments from one vector to another. The identity of the experimental vectors and construct were confirmed using restriction digestion and ligation experiments. The construct of test expression vector and control expression vectors were also checked and confirmed by sequencing method for insertion of required fragments in proper orientation.

(A) Position of intron

The intron syn7 was selected for IME strategy to overexpress the erythropoietin protein in *Nicotiana tabacum* plant. As it is already reported in literatures that intron works as a regulatory element for enhancement of expression of transgene, if it is inserted at the proper site with the gene (Rose 2002). There were three preferable or suitable sites mentioned in previous research.

i. Insertion of intron within 5′ UTR region of the gene
ii. Between promoter and coding region
iii. Within coding region

Based on this for the present study position of intron was decided between promoter and coding region.

(B) Isolation, purification, qualitative and quantitative analysis of plasmid DNA

All the plasmid vectors DNA used in the present study were isolated from transformed bacterial culture of *E. coli* using alkaline lysis method **(Section 3.2.2.2).** Purification of isolated plasmid DNA was done by phenol-chloroform method as mentioned in **Section 3.2.2.3.** Qualitative and quantitative analysis of DNA was performed using agarose gel electrophoresis method and spectrophotometric method **(Section 3.2.2.4).** Yield of all of the purified plasmid DNA was obtained in range of 0.92- 2.2 µg/µl. The purified plasmid DNA was subjected to restriction digestion and ligation for construction of desired control and test expression vectors.

(C) Restriction digestion of the purified plasmid DNA for the construction of desired vector

Restriction digestion is one most convenient and old technique for confirmation of the vector. Nowadays, there are five types of restriction endonuclease enzymes available. Among of these restriction enzymes type II has the capacity to recognize and cleave the DNA duplex at specific sites (Roberts 1976). So, this enzyme is widely used for the DNA cleavage.

In the present study this technique was used for the construction of desired vectors using various, type II restriction enzymes. During the restriction digestion experiment single and double digestion of purified plasmid DNA was carried out. Single digestion reaction was used as a control to assure that individual enzyme activity. One more control was also put containing plasmid DNA and restriction buffer except restriction enzyme to analyse the contamination or error in reaction mixture. Single digestion reaction and double digestion reaction were set as mentioned in **Table 3.1 and 3.2**. Restriction digestion reaction process was followed as mentioned in **Section 3.2.2.5.** Further, digested plasmid DNA was analysed on 0.8 or 1% agarose gel **(Section 3.2.2.4b)** and size of the DNA fragments was compared with the DNA marker.

(D) Ligation of restriction digested DNA fragment

Ligation is a part of molecular biology technique which is widely used to join the DNA fragments to construct the recombinant DNA using T4 DNA ligase enzyme (Sambrook and Russell 2007) T4 DNA ligase is used to join, nicked DNA duplex by the formation of phosphodiester bond between 5′-phosphate and 3′-hydroxyl termini in duplex DNA. It can ligate cohesive or "sticky" ends of DNA (Lohman et al. 2011). In the present study this ligation technique was used to construct the desired vector (control and test expression vectors). Plasmid DNA digested with the restriction enzymes were subjected to the ligation reaction as mentioned in **Section 3.2.2.6.** Digested DNA fragments of insert and backbone vectors were subjected to the ligation using ligase enzyme as mentioned in **Materials and Methods, Table 3.3.** The concentration or amount of both vectors was taken in the form of their molar ratio i.e. 1:3, 1:6. In ligation reaction, concentration of insert DNA was kept, 3 times higher than the vector backbone concentration to increase the probability of ligation of insert (fragment) in the vector backbone.

(E) PCR Screening of transformed bacterial clones

Selected transformed bacterial clones have been subjected to the PCR screening or analysis using transgene specific primers. In the present study, there are two transgenes i.e. cEPO and Syn7 present in the vectors. So, the confirmation of their presence in transformed bacterial colonies was carried out using specific forward and reverse primers **(Appendix VI).** PCR reaction was set as mentioned in **Section 3.2.4.1.** Transgene amplification specific reaction conditions for cEPO and Syn7 were set as mentioned in **Table 3.10**. Control reactions i.e. positive and negative controls were kept to assure the error free experiment. Further, PCR products were run on 2% agarose gel **(Section 3.2.2.4b)** and size of amplicon was compared with 50 bp DNA ladder.

(F) Construction of test expression vector

Test expression vector was constructed using backbone of pFGC5941 vector. In this vector, intron sequence along with cEPO sequence was inserted. Detailed construction strategy is as follow.

Strategy 1

- Excision of cEPO fragment from pPERDB33cEPO vector using *Nco*I and *BstE*II RE sites.
- Ligation of cEPO in pUC57Syn7 vector at downstream of intron (at same RE sites i. e. *Nco*I and *BstE*II) **(Figure 4.6).**

In all these steps, the confirmation of constructs was done using restriction digestion and ligation processes. Analysis of restricted digested and ligated fragments was carried out using agarose gel electrophoresis method.

Strategy 1

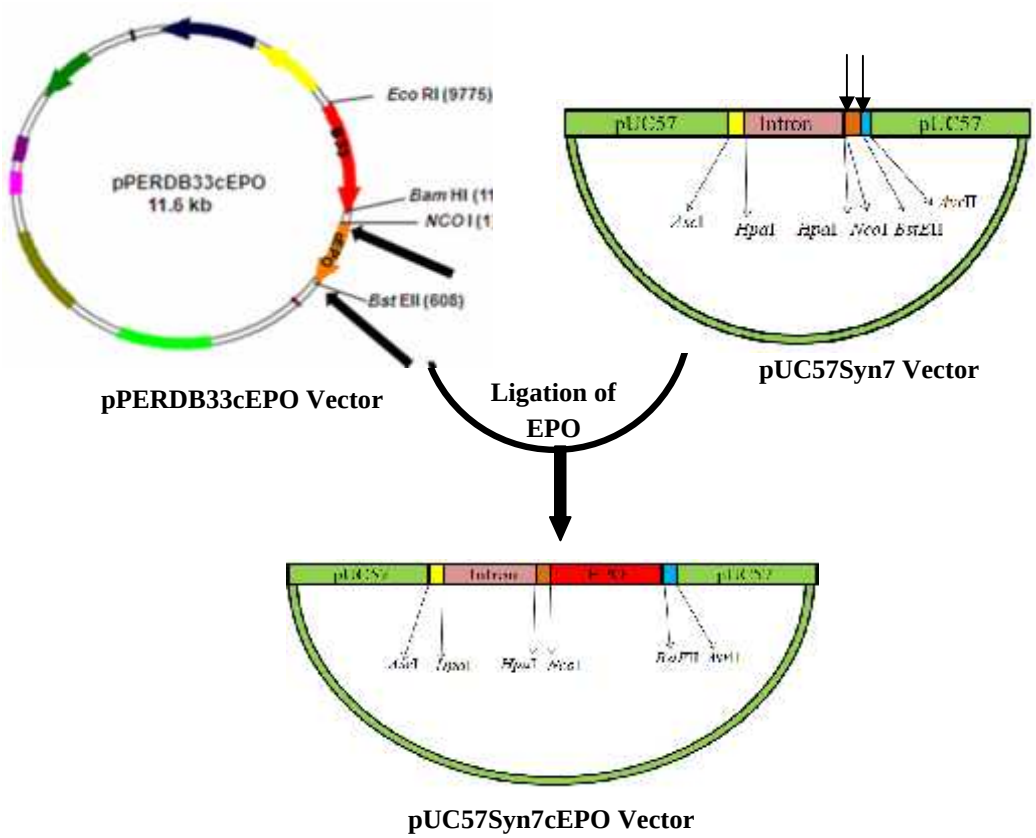


Figure 4.6: Schematic representation of insertion of cEPO into the pUC57Syn7 vector backbone.

Experimental part

4.1.3.1 Excision of cEPO from pPERDB33cEPO

Purified plasmid DNA, 1 µg was subjected to the double digestion using restriction enzymes *Nco*I and *Bst*EII for excision of cEPO fragment**.** To avoid any experimental error various controls (single digestion) namely- plasmid DNA digested with *Nco*I, plasmid DNA digested with *Bst*EII and plasmid DNA without any restriction enzymes were kept. After RE digestion, the samples were precipitated, air dried and dissolved in 10µl of tris-EDTA buffer of pH 8.0. Thereafter, all these samples were observed on 0.8 % agarose gel along with DNA marker. Results obtained showed that double digestion of plasmid DNA was resulted in excision of desired EPO fragment **(Figure 4.7, Lane 2)**. The size of the excised fragment (596 bp) was compared with marker present in **Lane 1** which confirms the band is of cEPO.

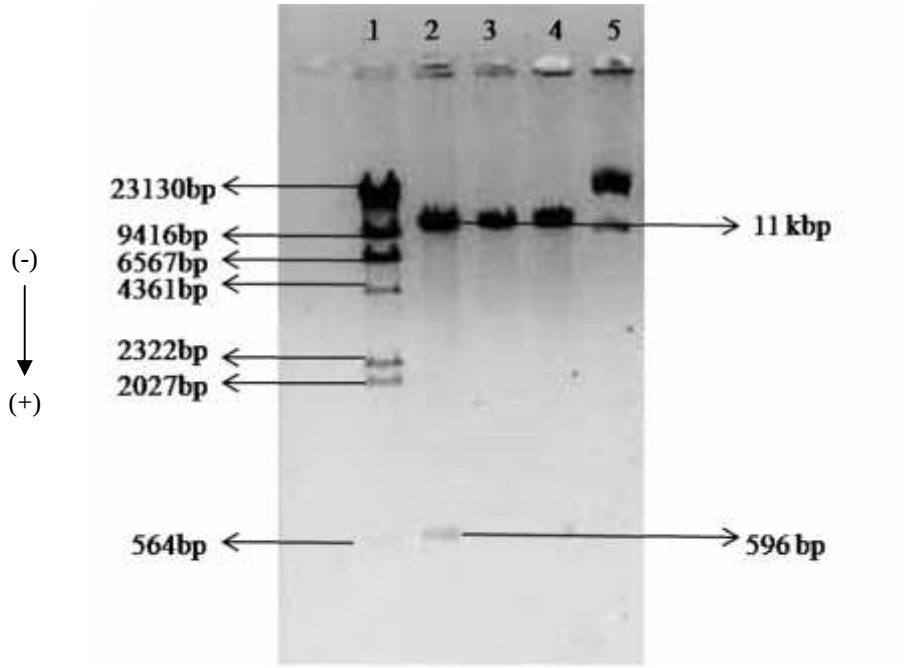


Figure 4.7: Restriction digestion of pPERDB33cEPO plasmid DNA
Lane 1: Lamda DNA marker
Lane 2: pPERDB33cEPO digested with *NcoI and BstEII*
Lane 3: pPERDB33cEPO digested with *NcoI*
Lane 4: pPERDB33cEPO digested with *BstEII*
Lane 5: Undigested pPERDB33cEPO (Control)

Single digested fragment of plasmid DNA present in **Lane 3 and 4** confirms the proper activity of RE enzymes individually, while undigested DNA present in **Lane 5** proves the use of right vector for RE digestion as well as it confirms the proper activity of all the chemicals used in the experiments.

4.1.3.2 RE digestion of pUC57Syn7 with *Nco*I and *BstE*II restriction enzymes

Purified DNA of pUC57Syn7 was also digested with same RE enzymes (*Nco*I and *BstE*II) to facilitate ligation of cEPO. 1 µg of plasmid DNA of pUC57Syn7 was subjected for double digestion. Three control reactions- two with single restriction enzyme individually and one without RE enzyme was kept during experimentation.

Results obtained as seen on agarose gel, double digested fragment of pUC57Syn7 with *Nco*I and *BstE*II in **Lane 2 (Figure 4.8)**. In double digestion reaction no excised fragment was observed on gel due to the smaller size of the excised fragments (around 5-6 bp). In **Lane 3**

and 4 of single digested linear DNA with *Nco*I and with *BstE*II were observed. Undigested plasmid DNA was also observed in **Lane 5**. The desired size of single digested and double digested plasmid DNA confirms that no experimental error occurred during experiments. Lambda DNA marker was used to compare the size of digested plasmid DNA **(Figure 4.8, Lane 1)**.

The double digested plasmid DNA was used to insert cEPO fragment obtained in previous experiment.

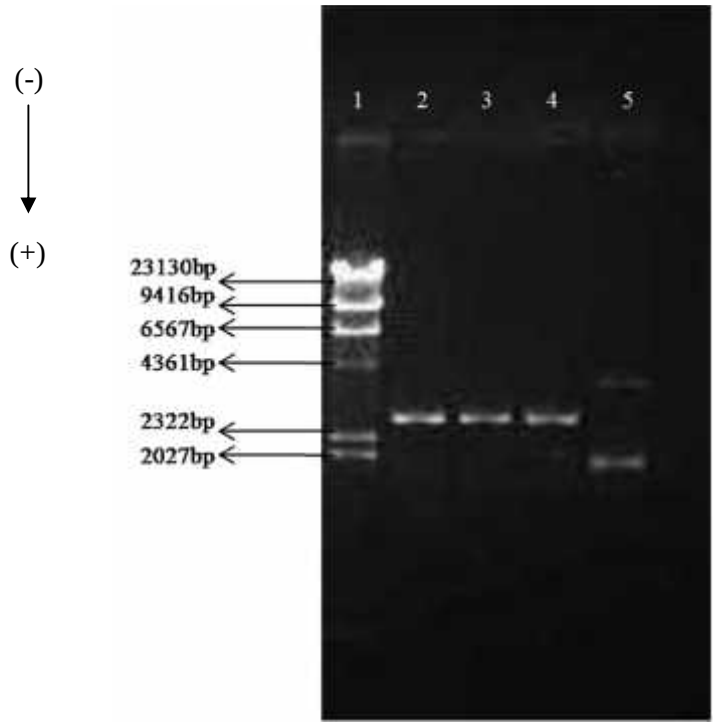


Figure 4.8: Restriction digestion of pUC57Syn7 plasmid DNA with *Nco*I and *BstE*II
Lane 1: Lambda DNA marker
Lane 2: Double digestion of pUC57Syn7 with *Nco*I and *BstE*II
Lane 3: Digested with *Nco*I
Lane 4: Digested with *BstE*II
Lane 5: Undigested pUC57Syn7

4.1.3.3 Ligation of cEPO into pUC57Syn7 & transformation of the *E. coli* DH5α cells with ligated product (pUC57Syn7cEPO)

After the restriction digestion of both the vectors were subjected to ligation reaction using ligase enzyme. Two different molecular weight ratio 1:3 and 1:6 of both the digested vectors

(Backbone vector: insert vector) were placed in the microfuge tube with ligase enzyme and ligase buffer. After ligation reaction, ligated product was used for the bacterial transformation. *E.coli* cells were transformed with ligated product using transformation method as mentioned in **Section 3.2.2.8a**. Transformed bacterial colonies carrying ligated pUCSyn7cEPO vector were selected on LA plate containing ampicillin antibiotic (1µg/1ml) **(Figure 4.9)** as amplicine resistant selection marker is present in the backbone of the pUC57Syn7 vector. Transformation efficiency was obtained 7.2 x 10^1 transformants/µg of DNA with 1:3 molecular ratio of the backbone vector and insert vector**.**

Figure 4.9: Transformed colonies of *E.coli* DH5α with pUC57Syn7cEPO vector

4.1.3.4 PCR confirmation of transformed clones

Appeared transformed colonies on ampicillin antibiotic selection plate were subjected to the colony PCR using EPO specific primers as ligation of cEPO fragment was expected in the vector. Total 9 colonies were individually subjected to PCR reaction for the amplification of EPO. The PCR amplified product was analysed on 2% agarose gel **(Figure 4.10)** under Gel documentation unit. According to the primer designing of cEPO, expected amplicon size of cEPO was 508 bp which was observed on gel in **Lane 1, 2, 3, 8 and 9.** The results of the experiment showed that out of randomly selected 9 ampicillin resistance bacterial colonies only 5 bacterial colonies have plasmid vector pUC57Syn7 inserted with cEPO fragment the other 4 baterial colonies selected on plate carries self ligated pUCsyn7 vector as it carries ampicillin resistance gene. Positive control and negative control reaction were also kept during experimentation. Plasmid DNA of pPERDB33cEPO was subjected to the reaction mixture for positive control and no DNA was subjected to the negative control reaction

mixture. In positive control, amplicon of 508 bp of cEPO was observed in **Lane 5** and no amplicon was observed in negative control in **Lane 11**. These all controls of PCR reaction assured that PCR was performed without any experimental error. The size of amplicon was confirmed and compared with the GeneRuler 50 bp DNA ladder (Fermentas, India). pUC57Syn7 vector obtained after ligation of cEPO in this experiment was named pUC57Syn7cEPO.

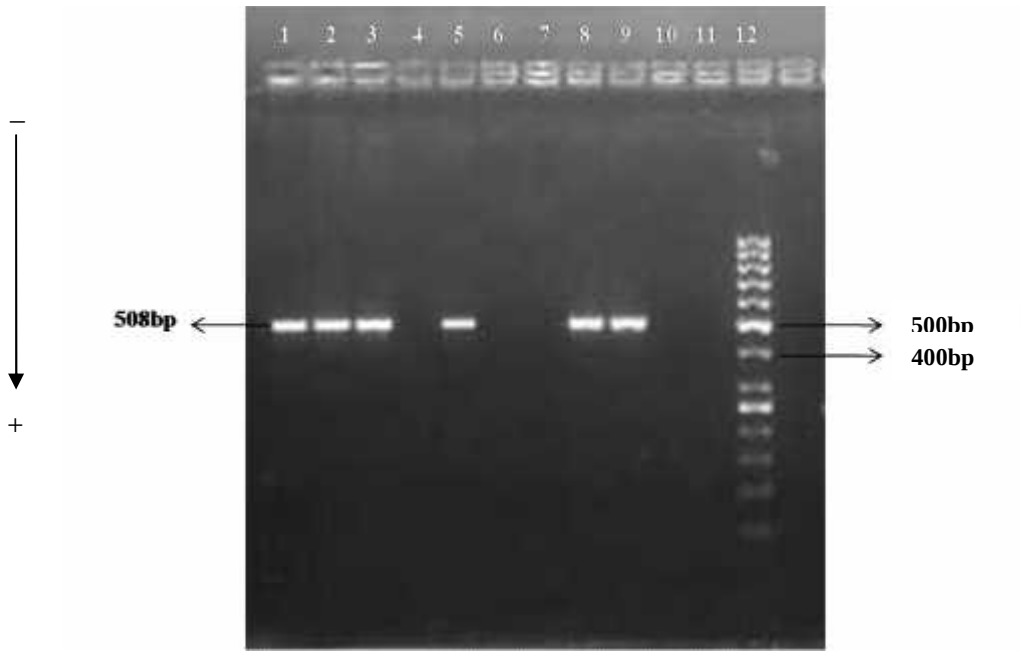


Figure 4.10: PCR analysis of amplified PCR product of cEPO.
Lane 1-4: Colonies; **Lane** 5: Positive control (Plasmid DNA);
Lane 6-10: Colonies; **Lane** 11: Negative control;
Lane 12: 50 bp ladder

Bacterial colonies carrying pUC57Syn7cEPO vector (PCR positive) were individually grown in 2 ml LB medium containing ampicillin antibiotic in shaker at 180 rpm for 16 h at 37^{o}C. Hundred microlitre of grown culture was inoculated in 10 ml medium containing ampicillin antibiotic and grown under the above mentioned conditions. Further confirmation of clones was also done using restriction enzyme digestion technique. As cEPO fragment was ligated in the pUC57Syn7 vector at the *Nco*I and *BstE*II RE sites, the same RE enzymes were used to excise cEPO fragment to confirm of the presence of cEPO fragment in the pUC57Syn7 vector.

4.1.3.5 RE confirmation of pUC57Syn7cEPO using *Nco*I and *Bst*EII restriction enzymes

Isolated plasmid DNA (pUC57Syn7cEPO) from the transformed colonies were further confirmed by restriction digestion using *Nco*I and *Bst*EII restriction enzymes as these RE sites were present at the both ends 5′ and 3′ of the cEPO fragment respectively in vector. 1µg of DNA was subjected to both single digestion and double digestion. RE products were observed on 0.8 % agarose gel. Expected and excised fragment of 596 bp of cEPO and 2.9 kb fragment of backbone vector was observed in double digested reaction product which was observed in **Lane** 2 **(Figure 4.11).** In other single digested (with only *Nco*I and with only *Bst*EII RE enzymes) control reactions, expected fragment size (3.5 kb) was observed in **Lane** 3 and **Lane** 4. In control reaction, undigested DNA was observed as it is. Size of fragments was compared with the lambda DNA ladder in **Lane** 1. These all control revealed that there was no experimental error.

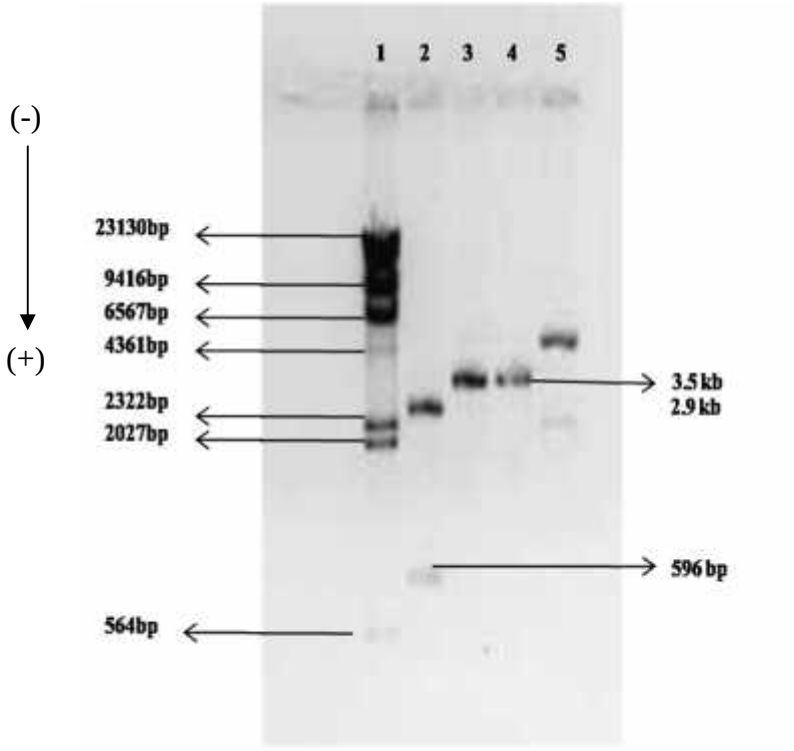


Figure 4.11: Double digested plasmid DNA of pUC57SynEPO with *NcoI* and *BstEII*.
Lane 1: Lambda DNA marker
Lane 2: Double digestion of pSynEPO with *NcoI* and *BstEII*
Lane 3: pUC57SynEPO digested with *NcoI*
Lane 4: pUC57SynEPO digested with *BstEII*
Lane 5: Undigested pUC57SynEPO (control)

After the RE confirmation of pUC57Syn7cEPO vector, strategy 2 was followed for excision of whole cassette (syn7 intron and cEPO fragments) from pUC57Syn7cEPO vector and insertion of cassette in pFGC5941 expression vector.

Strategy 2

- Excision of Syn7cEPO sequence from pUC57Syn7cEPO vector using *Asc*I and *Avr*II RE sites.
- RE digestion of pFGC5941 with *Asc*I and *Avr*II RE enzymes.
- Ligation of both RE digested vectors using ligase enzyme to facilitate the insertion of whole cassette Syn7cEPO fragment in pFGC5941 vector between promoter CaMV35S and terminator OCS3′ in pFGC5941 vetor **(Figure 4.12).**

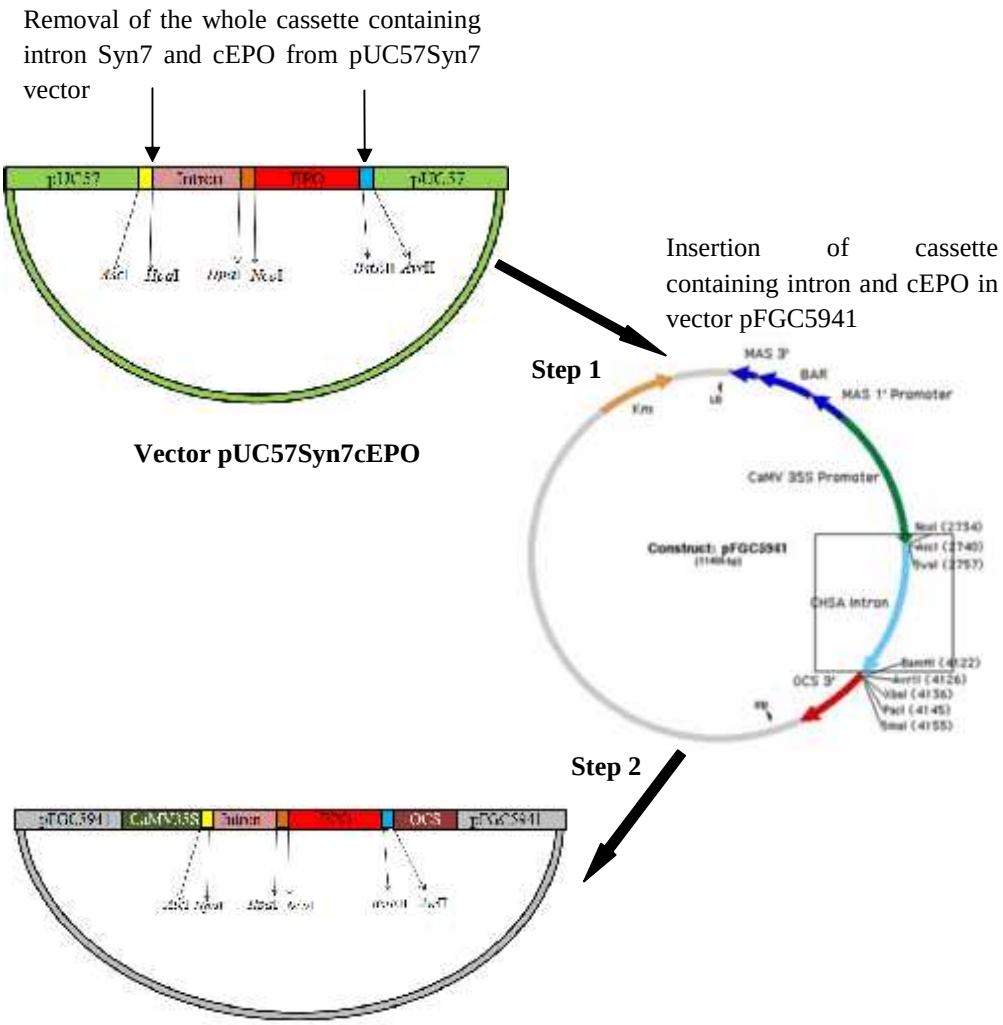


Figure 4.12: Schematic representation of construction of final expression vector pFGC5941Syn7cEPO

Experimental Part

4.1.3.6 Excision of cassette containing Syn7 and cEPO from pUC57Syn7cEPO vector using *Asc*I and *Avr*II RE enzymes

The isolated and purified pUC57Syn7cEPO plasmid DNA from *E. coli* bacterial colonies obtained in previous experiment **(Section 4.1.3.3)**, was subjected to the restriction digestion with *Asc*I and *Avr*II RE enzymes. The experiment was conducted along with single digestion of plasmid DNA with both the enzymes individually and plasmid DNA without restriction enzyme. RE digested products were observed on 0.8% agarose gel. Double digestion in RE reaction was expected to give 2 fragments, one fragment of Syn7cEPO of 850 bp (Syn7 246 bp and cEPO 596 bp) and another fragment of pUC57 vector backbone 2.7 kb. Presence of both these fragments on agarose gel **Lane 4 (Figure 4.13)** confined the successful restriction digestion of the vector. Desired size of vectors present in **Lane 1, 2 and 3** ensure error free experimentation. Size of the obtained DNA fragments was determined with comparison of DNA marker in **Lane 5.**

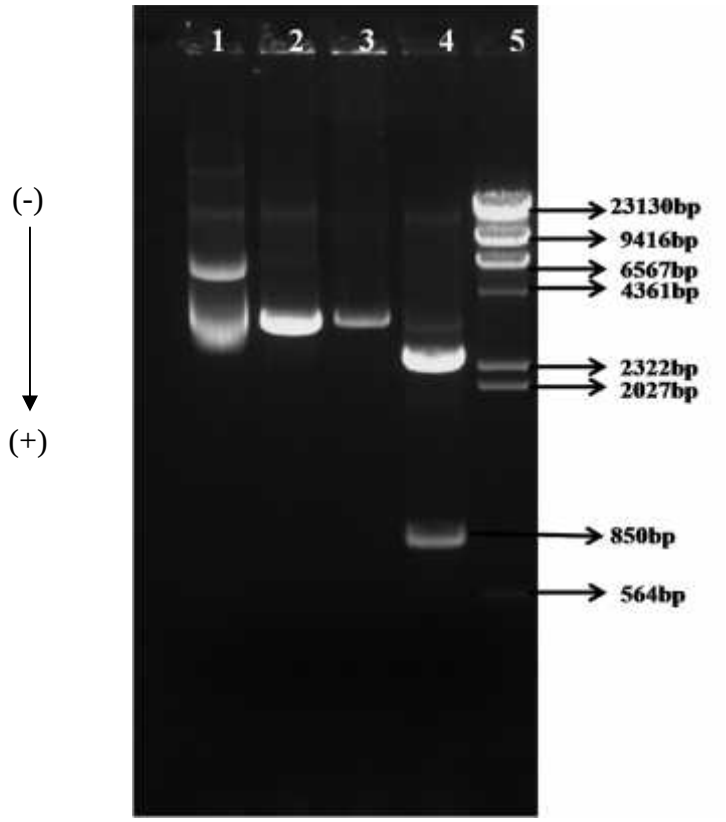


Figure 4.13: Restriction digestion of pUC57Syn7cEPO with *Asc*I and *Avr*II enzymes.
Lane 1: Undigested plasmid DNA of pUC57Syn7cEPO (Control)
Lane 2: pUC57Syn7cEPO digested with *Asc*I
Lane 3: pUC57Syn7cEPO digested with *Avr*II
Lane 4: pUC57Syn7cepo digested with *Asc*I and *Avr*II
Lane 5: Lambda DNA marker

4.1.3.7 Restriction digestion of pFGC5941 vector with *Asc*I and *Avr*II

Purified plasmid DNA of pFGC5941, 1µg was subjected to double restriction digestion using *Asc*I and *Avr*II enzymes. Various controls such as single digestion of DNA with *Asc*I and *Avr*II enzyme individually and without enzyme were also kept. The results obtained as visualised on 0.8% agarose gel showed expected fragments of 1385 bp of CHSA intron present in the pFGC5941 vector and backbone fragment of 10 kb in **Lane 2 (Figure 4.14).** In all the control samples desired and expected size of fragments were observed in **Lane** 3 (digested with *Asc*I) and **Lane** 4 (digested with *Avr*II). Undigested DNA was also observed in **Lane** 5. Size of all the fragments was compared with lambda (λ) DNA marker.

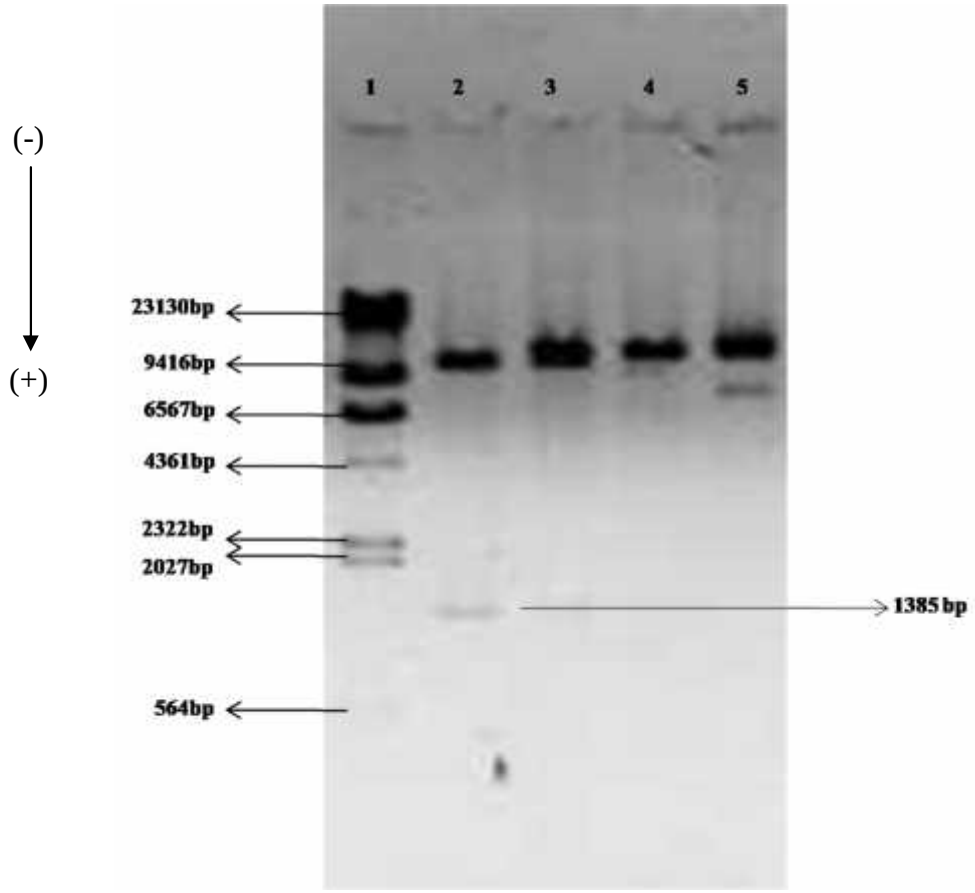


Figure 4.14: Agarose gel picture of digested plasmid DNA of pFGC5941 with *AscI* and *AvrII* restriction enzymes.
Lane 1: Lamda DNA marker Undigested pFGC5941 (control)
Lane 2: pFGC5941 digested with *AscI* and *AvrII*
Lane 3: pFGC5941 digested with *Asc*I
Lane 4: pFGC5941 digested with *AvrII*
Lane 5: Undigested pFGC5941

Both the vectors i.e. pUC57Syn7cEPO and pFGC5941 were digested with the same RE enzymes i.e. *Asc*I and *Avr*II. Both the digested vectors had generated the same RE ends after digestion which made the vector or gene compatible for the ligation.

4.1.3.8 Ligation of Syn7cEPO DNA fragment in pFGC5941 vector

Both the RE digested products obtained in previous experiments **(Section 4.1.3.6 and 4.1.3.7)** were further subjected to the ligation using ligase enzyme. Two different molecular weight ratio 1:3 and 1:6 of backbone vector to insert vector were taken and ligation reaction was set (**Table 3.3)**. Ligated products obtained after lgation reaction were further transformed in to *E.*

coli DH5α cells using calcium chloride heat shock method **(Section 3.2.2.8a)** and transformants were selected on kanamycin (1µg/1ml) containing luria agar plate **(Figure 4.15)** as kanamycin resistant gene is present in vector pFGC5941 vector backbone which facilitate selection of transformed bacterial cells containing pFGC5941 vector containing desired gene. Transformation efficiency was obtained 4.12 x 10^2 transformants/µg of DNA with 1:3 molecular ratio of backbone and insert vector. The appeared transformed colonies were further subjected to the PCR screening for the confirmation of desired gene with gene specific primers.

Figure 4.15: Transformed colonies of *E. coli* DH5α transformed with pFGC5941Syn7cEPO

4.1.3.9 PCR confirmation of transformed clones

Total 7 randomly picked colonies from the appeared transformed colonies on kanamycin antibiotic selection plate were subjected to the colony PCR using cEPO specific primers. Positive control (plasmid DNA of pUC57Syn7cEPO) and negative control reactions (No DNA in the reaction mixture) were also kept along with the test experiment. The PCR amplified product was analysed on 2% agarose gel **(Figure 4.16).** Out of 7 colonies only three colonies showed amplicon of 508 bp of cEPO as visualized on gel in **Lane 4, 5 and 8** which confirmed the presence of cEPO in vector pFGC5941. The other appeared colonies must be the containing pFGC5941 self ligated vector. Amplicon of 508 bp was observed in positive control **Lane 2** and no amplicon was observed in negative control **Lane 3**.

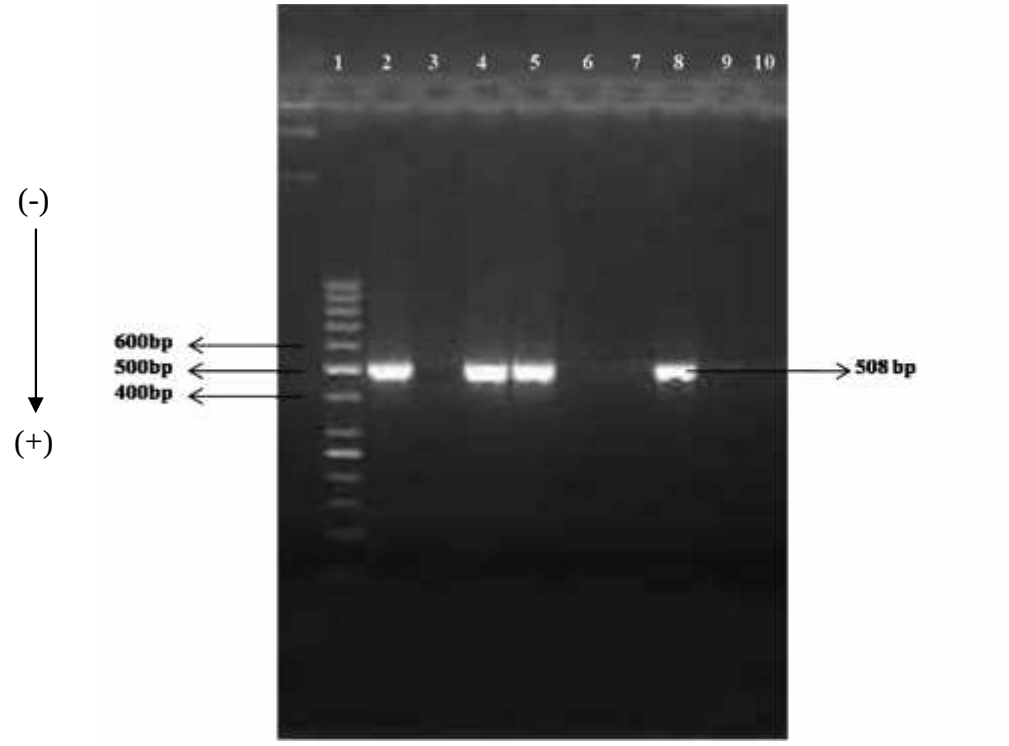


Figure 4.16 PCR of transformed clones of *E. coli* with pFGC5941Syn7cEPO using EPO specific primer
Lane 1: 50 bp DNA ladder, **Lane** 2: Positive control, **Lane** 3: Negative Control, **Lane** 4-10: kanamycin resistance Colonies

PCR positive clones represented successful ligation of whole cassette of the Syn7 and cEPO in the pFGC5941 vector. After the confirmation of the PCR positive clones, individual clone was grown in 2 ml LB medium containing kanamycin antibiotic in shaker at 180 rpm for 16 h at 37^{o}C and then 100 µl of this 16 h grown culture was inoculated in 10 ml medium containing kanamycin antibiotic and grown under the above mentioned conditions. Further confirmation of clones was done using restriction enzyme digestion of isolated plasmid from PCR confirmed positive clones. As Syn7 and cEPO fragment was ligated in the pFGC5941 vector. The vector was named as **pFGC5941Syn7cEPO**. The same RE enzymes *Asc*I and *Avr*II were used to excise the whole cassette of Syn7cEPO from the vector for the confirmation of the presence of Syn7cEPO fragment (850 bp) in the pFGC5941 vector.

4.1.3.10 RE confirmation of vector pFGC5941Syn7cEPO with *Asc*I and *Avr*II enzymes

Purified DNA, 1 µg isolated from pFGC5941Syn7cEPO vector transformed bacterial colony was subjected to double restriction digestion with *Asc*I and *Avr*II restriction endonuclease enzymes. Single digestion reaction of plasmid with both the enzymes and plasmid without enzyme reactions were kept as control. Excised fragment of 850 bp (containing Syn7 of 246 bp and cEPO of 596 bp) from double digested plasmid was observed in **Lane 1 (Figure 4.17)** on 0.8% agarose gel. In single digested control samples (*Asc*I or *Avr*II) expected fragments of 11.9 kb size were observed in **Lane 2** (digested with *Asc*I) and **Lane 3** (digested with *Avr*II). Undigested control DNA of 11.9 kb size was also observed in **Lane 4.** These results confirm the transformed bacterial colony selected on agar plate contains pFGC5941syn7cEPO vector and also RE digestion experiment was performed without any error. Size of fragments was compared with lambda DNA/*Hind*III marker in **Lane 5 (Figure 4.17).**

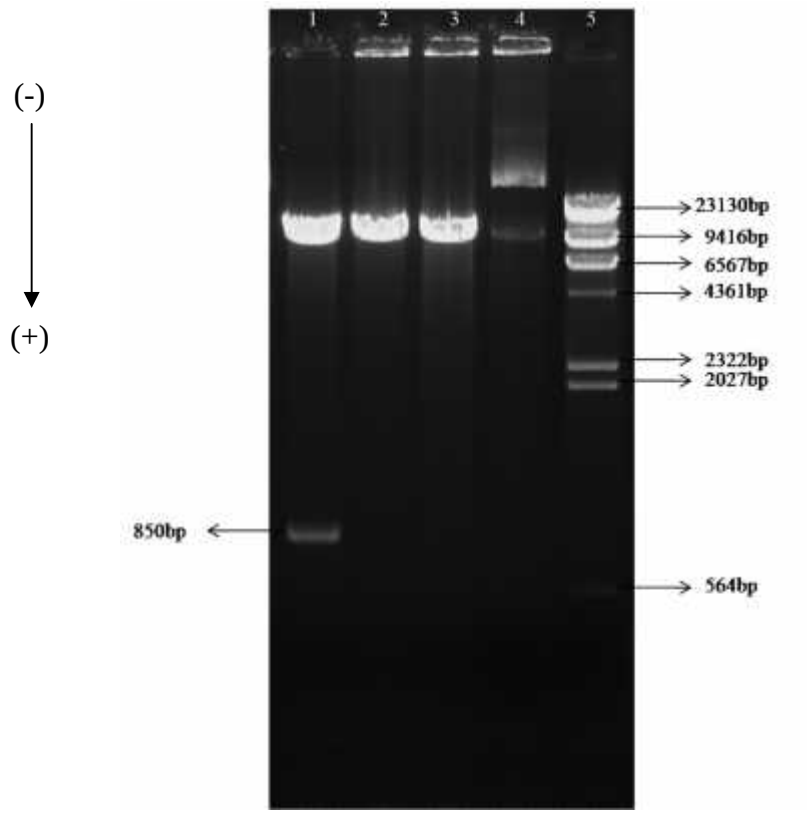


Figure 4.17: Restriction digestion of pFGC5941Syn7cEPO with *Asc*I and *Avr*II.
Lane 1: pFGC5941Syn7cEPO digested with *Asc*I and *Avr*II (double digestion)
Lane 2: pFGC5941Syn7cEPO digested with *Asc*I
Lane 3: pFGC5941Syn7cEPO digested with *Avr*II
Lane 4: Undigested DNA (Control)
Lane 5: Lambda DNA/*Hind*III marker

All these experiment following vector construction strategy were resulted in successful construction of '**expression vector pFGC5941Syn7cEPO**'. To ensure that desired vector was constructed as per *in silico* design, the constructed vector was subjected to sequencing before its use for any genetic transformation study.

4.1.3.11 Sequencing of constructed vectors

Confirmation of test expression vector pFGC5941Syn7cEPO was done by sanger sequencing method using four capillary 3130 sequencer (Applied Biosystem, ABI) **(Figure 4.18)**

The primers were designed to amplify the entire cloning cassette containing Syn7 and cEPO as well as the flanking sequence of the vector (primer sequences mentioned in **Appendix VI**). These primers were used for the amplification of the particular constructed chimera part of test expression vectors using PCR method. The PCR amplified products of the vectors were subjected to the sequencing.

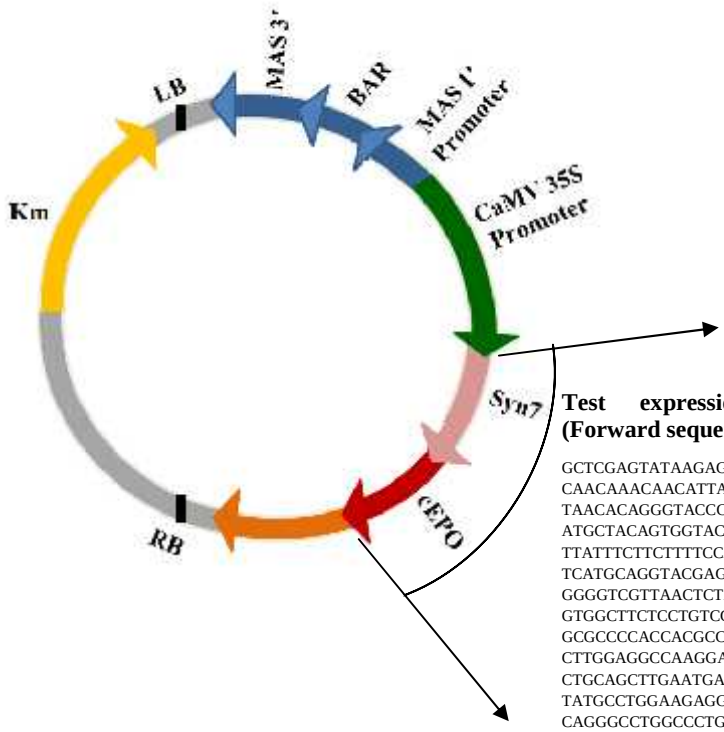


Figure 4.18: Sequenced part of test expression vector pFGC5941Syn7cEPO

Test expression vector pFGC5941Syn7cEPO (Forward sequence)

GCTCGAGTATAAGAGCTCTATTTTTACAACAATTACCAACAACAACAAA
CAACAAACAACATTACAATTACATTTACAATTACCATGGGGCGCGCCGT
TAACACAGGGTACCCGGGGATCCTCTAGAGCCACGTCCCTCATGTCGAC
ATGCTACAGTGGTACCACTCGTGAGGTAAGATTATCGATATTTAAATTAT
TTATTTCTTCTTTTCCATTTTTTTGGCTAACATTTTTCATGGTTTTATGATA
TCATGCAGGTACGAGCGCTCGAGATGGGTATCGCGATAGGCCTGCTGCA
GGGGTCGTTAACTCTAGACCATGGGGGTGCACGAATGTCCTGCCTGGCT
GTGGCTTCTCCTGTCCCTGCTGTCGCTCCCTCTGGGCCTCCCAGTCCTGG
GCGCCCCACCACGCCTCATCTGTGACAGCCGAGTCCTGGAGAGGTACCT
CTTGGAGGCCAAGGAGGCCGAGAATATCACGACGGGCTGTGCTGAACA
CTGCAGCTTGAATGAGAATATCACTGTCCCAGACACCAAAGTTAATTTC
TATGCCTGGAAGAGGATGGAGGTCGGGCAGCAGGCCGTAGAAGTCTGG
CAGGGCCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCAGGCCCTGT
TGGTCAACTCTTCCCAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAA
AGCCGTCAGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGGGA
GCCCAGGAGAAGCCATCTCCCCTCCAGATGCGCTCAGCTGCTCCACTCC
GAAACAATCACTGCTGACACTTTCGGCAACTCTCCGAGTCTACTTCATTC
CTCGGGGAAGCTGAAGCTGTACA

The obtained nucleotide sequence was further subjected to multiple alignment using CLUSTALW software, the results of sequence alignment showed that the sequence of vector was similar to the designed sequence of the constructed vector.

Confirmed plasmid DNA of the pFGC5941Syn7cEPO was further used for the transformation. *A. tumefaciens* cells were transformed with pFGC5941Syn7cEPO vector using electroporation technique.

4.1.3.12 Transformation of *A. tumefaciens* LBA4404 cells

Competent cells of *A. tumefaciens* prepared as per the protocol **(Section 3.2.2.8b, i)** were subjected to electroporation with 1µg/ µl of plasmid DNA of pFGC5941Syn7cEPO following methodology as per **Section 3.2.2.8b, ii.** Transformed colonies were selected on luria agar plates having antibiotics kanamycin (50 mg/L) and rifampicin (50 mg/L) (**Figure 4.19)** as kanamycin resistance gene is present in the backbone of the plasmid DNA and rifampicin resistance selection marker gene is present in the chromosomal DNA of the *A. tumefaciens.* So, both the antibiotics were used for the selection of the transformed cells of *A. tumefaciens* LBA4404 cells. Transformation efficiency was calculated as $4.11x10^2$ transformants/µg of DNA with the molecular ratio of 1:3 of the backbone vector and insert vector.

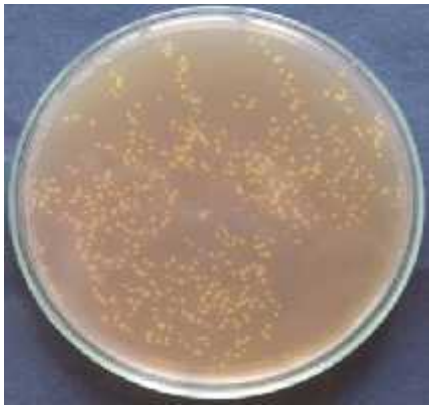


Figure 4.19: Transformed colonies of *A. tumefaciens* with pFGC5941Syn7cEPO vector

4.1.3.13 Screening of transformed *Agrobacterium* cells with test expression vector pFGC5941Syn7cEPO

Randomly picked 3 transformed colonies of *A. tumefaciens* were subjected to colony PCR with cEPO and Syn7 specific primers individually**.** PCR reactions and conditions for amplification of cEPO and Syn7 were set as mentioned in **Table 3.10**. After PCR amplification amplicons of cEPO (508 bp) and Syn7 (391 bp) were visualised on 2% agarose gel in **Lane 7-9** and **Lane 2-4** respectively **(Figure 4.20).** Isolated DNA of pFGC5941Syn7cEPO was amplified as positive control and PCR reaction mixture without any DNA was used as negative control. Size of amplicons was compared with the GeneRuler 50 bp DNA ladder (Fermentas, USA). No amplification was observed in the negative control **Lane 1** and **11** whereas amplicon of Syn7 and cEPO were observed in the both positive control lane in **Lane 5** and **10** respectively as its reaction mixture had the pure plasmid DNA **(Figure 4.20).**

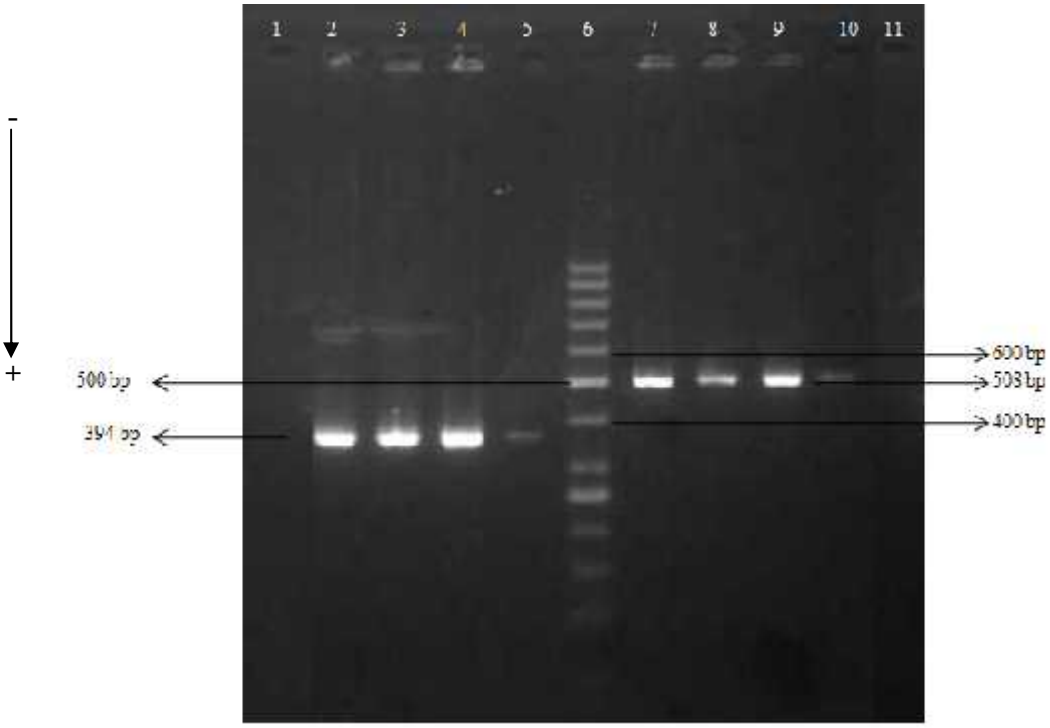


Figure 4.20: PCR of transformed clones of *A. tumefaciens* with pFGC5941Syn7cEPO using Syn7 and cEPO specific primers
Lane 1: Negative control
Lane 2- 4: Syn7 amplicon
Lane 5: Positive control
Lane 6: 50bp DNA ladder
Lane 7: 7-9 cEPO amplicon
Lane 10: Positive control
Lane 11: Negative control

Primer sequences for Syn7 intron were designed by covering the small flanking region of intron in vector. Resulting, if intron is present in the vector, then PCR amplified product will be 394 bp in size and if intron is absent in the vector, then PCR amplified product will be of 160 bp only.

Colony PCR screening of the transformed clones of *A. tumefaciens* cells confirmed the successful transformation of cells with the test expression vector. Further, these transformed *Agrobacterium* cells were used for the *Agrobacterium*-mediated genetic transformation of *N. tabacum* plant.

(D) Construction of control expression vector

Construction of control vector required the removal of intron sequence from the final expression vector pFGC5941Syn7cEPO (containing cEPO and Syn7 intron sequences). pFGC5941Syn7cEPO vector containing whole cassette (cEPO and Syn7 intron) was digested with *Hpa*I restriction enzyme as this RE sites were present at both ends of the intron **(Figure 4.21).** Digested RE product was run on agarose gel and desired backbone fragment (having cEPO only) was eluted and subjected to the self ligation using ligase enzyme. **(Figure 4.21).**

Strategy 3

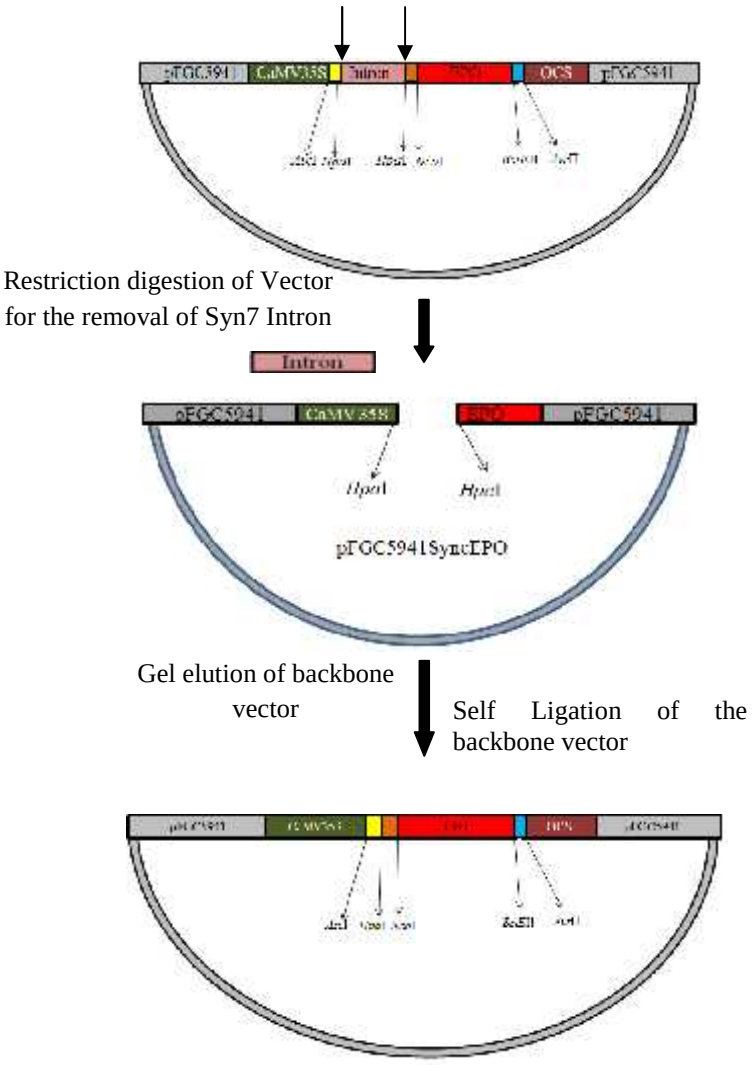


Figure 4.21: Schematic representation of construction of control expression vector pFGC5941cEPO

Experimental part

4.1.3.14 Restriction digestion of pFGC5941Syn7cEPO with *HpaI* enzyme

pFGC5941Syn7cEPO vector was subjected to the restriction digestion with *Hpa*I enzyme (depicted in **Figure 4.21**). Restriction digestion experiment was resulted in two DNA fragments of 10.6 kb and 246 bp, these fragments represent the backbone of pFGC5941Syn7cEPO and Syn7 respectively **(Lane 3, Figure 4.22).** Size of DNA fragments was compared with 50 bp DNA ladder (for small fragment) and λ DNA ladder (for large fragment). Undigested plasmid DNA was used as control (Digested DNA samples were observed on 1% agarose gel).

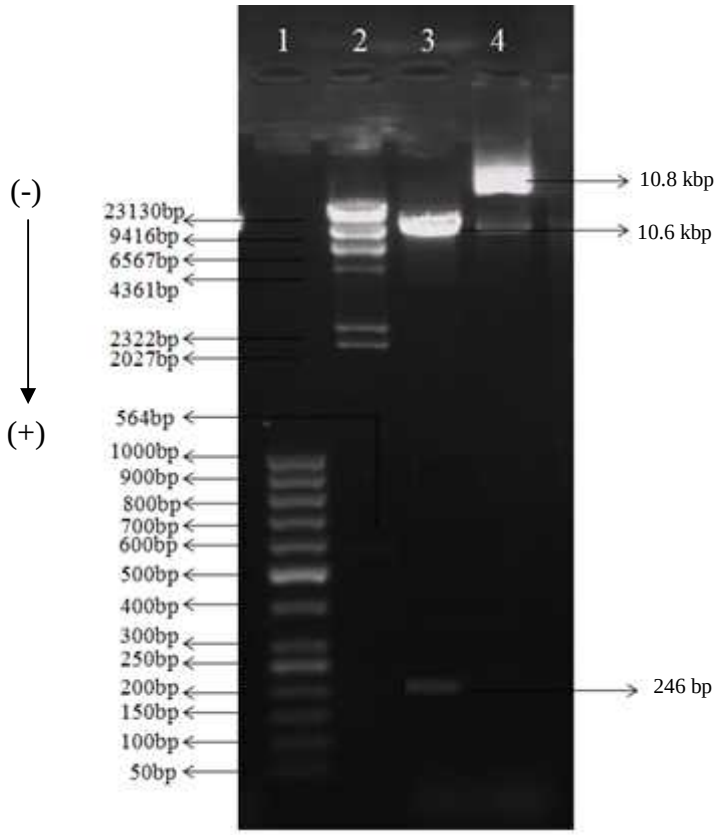


Figure 4.22: Restriction digestion of final expression vector (pFGC5941SyncEPO) with *HpaI* restriction enzyme,
Lane 1: 50 bp DNA ladder
Lane 2: Lambda DNA ladder
Lane 3: pFGC5941SyncEPo digested with *HpaI*
Lane 4: Undigested pFGC5941SyncEPO (control)

4.1.3.15 Gel elution of backbone of the vector from agarose gel

DNA fragment of 10.6 kb separated on agarose gel after restriction digestion **(Section 4.1.4.4)** was subjected to gel elution using Gel elution kit (sigma Aldrich, USA) (**Section 3.2.2.7).** Eluted fragments were further subjected to the ligation reaction.

4.1.3.16 Ligation of RE product and transformation in *E. coli* cells

Eluted backbone vector of previous experiment was subjected to self ligation using ligase enzyme (protocol mentioned in **Section 3.2.2.6** and **Table 3.3**) **(Figure 4.21**). The self ligated product was further used to transform bacterial cells. Transformation of *E. coli* DH5α cells with ligated product was performed using calcium chloride (chemical) method **(Section 3.2.2.8a)**.Transformed colonies were appeared on kanamycin luria agar plate. Transformation efficiency was calculated as 6.2 x 10^1 transformants/ µg of DNA with molecular ratio 1:6 of the backbone vector to insert vector **(Figure 4.23).**

Figure 4.23: Transformed colonies of *E.coli* DH5α cells with pFGC5941cEPO vector

4.1.3.17 PCR screening of transformed clones

Transformed colonies appeared on the kanamycin selection plate were further confirmed by colony PCR method. The vector (self ligated backbone) which was constructed in previous step is the control vector having only cEPO. Three transformed colonies were subjected to the PCR using cEPO specific primer. Results of experiment on 2% agarose gel showed, amplicon of 508 bp of cEPO in **Lane 4, 5 and 6** which confirmed that cEPO is present in the

transformed colonies. Negative control (no DNA or colony) and positive control (plasmid DNA of pFGC5941Syn7cEPO) were used to make experiment error free. No amplification was observed in negative control reaction in **Lane 3** while amplicon of 508 bp was observed in the positive control reaction in **Lane 2**. Size of the amplicon was compared with the 50 bp DNA ladder **(Figure 4.24)**.

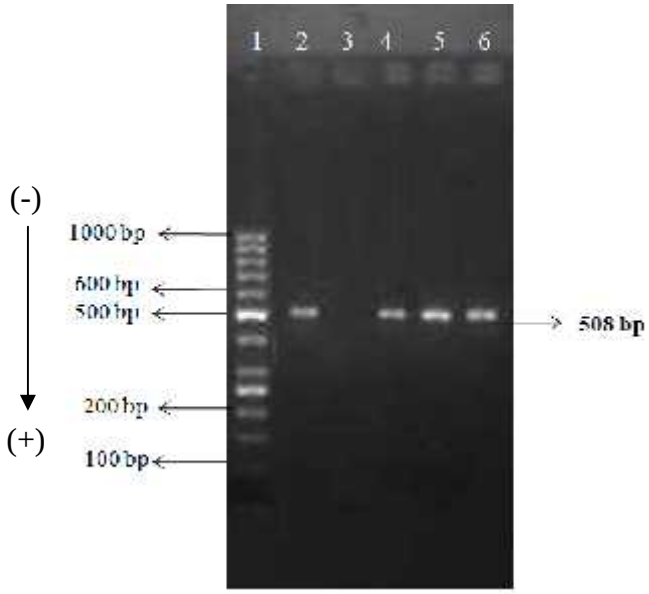


Figure 4.24: PCR confirmation of transformed clones of *E.coli* with control vector pFGC5941cEPO using EPO specific primer. **Lane**1- 50 bp DNA ladder; **Lane**2- positive control; **Lane**3 negative control; **Lane** 4-6- transformed colonies.

PCR positive clones were individually grown in 2ml LB medium containing kanamycin antibiotic in shaker at 180 rpm for 16 hr at 37^{o}C and then 100 µl of this culture was inoculated in 10 ml medium containing kanamycin antibiotic and grown under the above mentioned conditions. Further confirmation of clones was done using restriction enzyme digestion of isolated plasmid using *Asc*I and *Avr*II RE enzymes.

4.1.3.18 RE confirmation of vector with *Asc*I and *Avr*II

Isolated and purified plasmid DNA was subjected to the RE digestion using *Asc*I and *Avr*II RE enzymes as these RE enzymes were present at the 5′ (*Asc*I) end and 3′ (*Avr*II) end of the whole cassette which contained intron and cEPO in test expression vector. However, the fragment of intron had been already removed from test expression vector to construct the

control expression vector. So, after the digestion of the control expression vector with *Asc*I ans *Avr*II only cEPO fragment of 596 bp was observed on gel which confirmed that control expression vector had only cEPO. Size of the fragment was compared with the lambda DNA marker **(Figure 4.25)**. Required controls were used in the experiment.

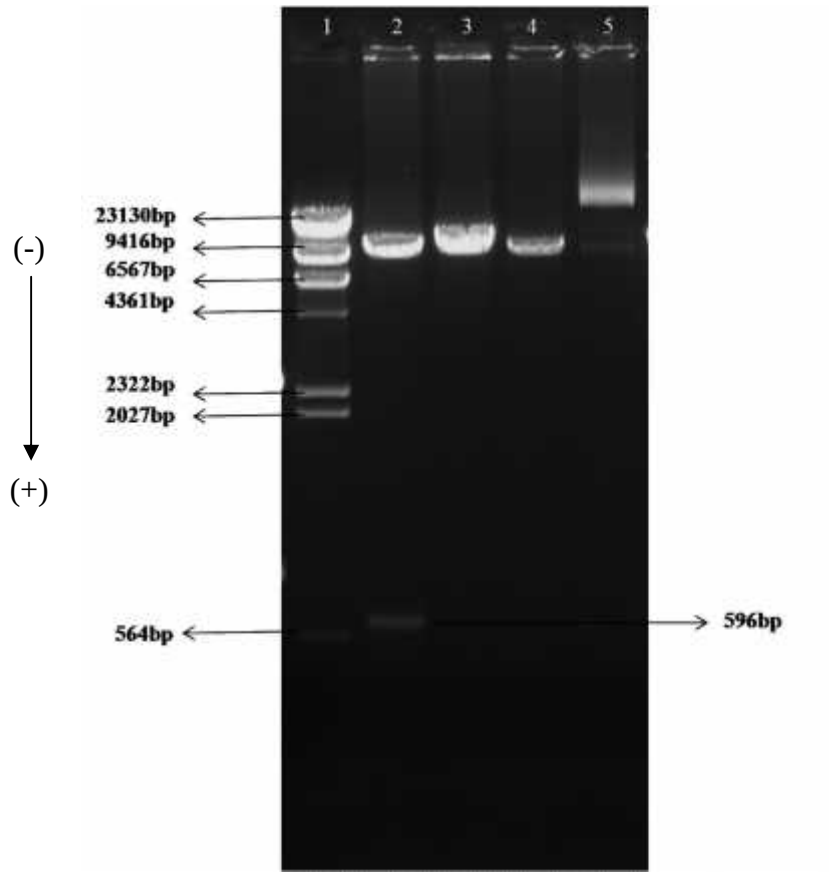


Figure 4.25: Agarose gel picture of digested plasmid DNA of pFGC5941cEPO with *Asc*I and *Avr* II

Lane 1: Lambda DNA marker
Lane 2: pFGC5941cEPO with *Asc*I and *Avr* II
Lane 3: pFGC5941cEPO digested with *Asc*I
Lane 4: pFGC5941cEPO digested with *Avr* II
Lane 5: Undigested pFGC5941cEPO plasmid DNA

After the confirmation of the vector by colony PCR and RE confirmation it was named as **pFGC5941cEPO vector** the '**control expression'** vector. Final confirmation of the control expression vector was done by sequencing technique.

4.1.3.19 Sequencing of the control expression vector

Confirmation of constructed control expression vector was done by sanger sequencing method using four capillary 3130 sequencer (Applied Biosystem, ABI) **(Figure 4.26)**

The primers were designed to amplify the entire cloning cassette as well as the flanking sequence of the vector. These primers were used for the amplification of the particular constructed chimera part of the control expression vectors. The PCR products of the vectors was subjected to the sequencing.

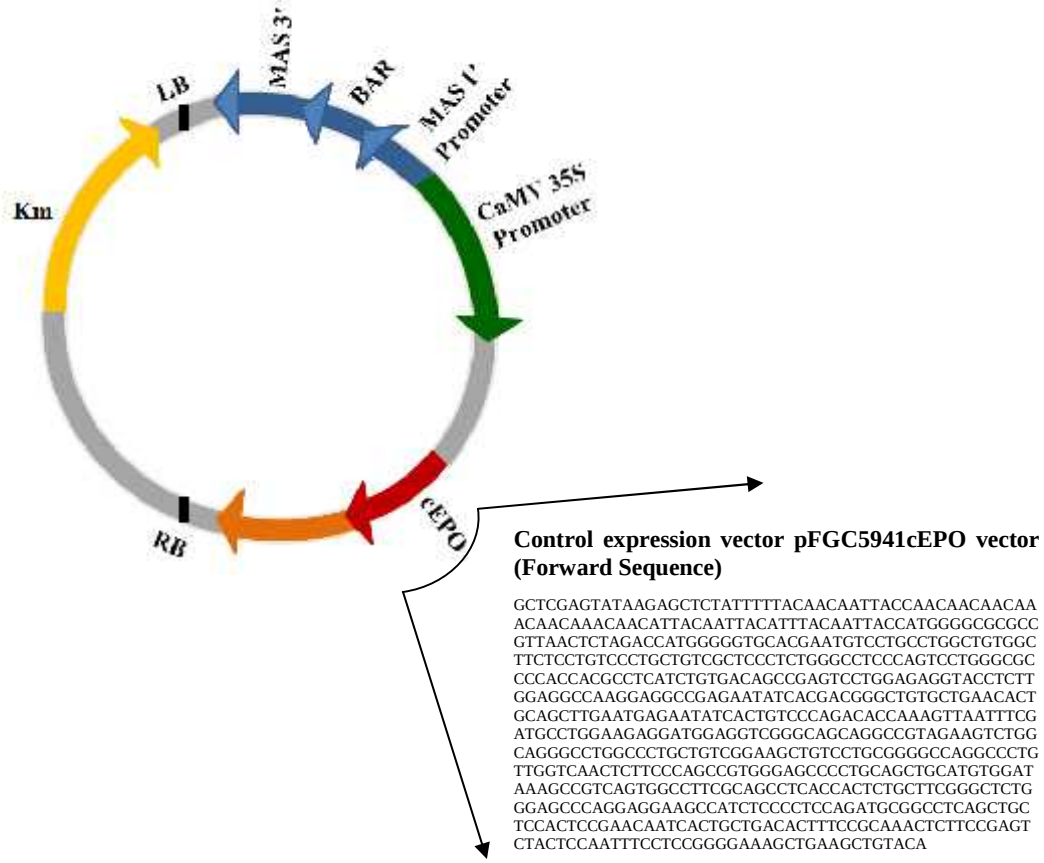


Figure 4.26: Sequenced part of control expression vector pFGC5941cEPO

The obtained sequence was subjected to multiple sequence alignment using CLUSTALW software which showed the absence of intron sequence in the control expression vector during analysis **(Appendix VII).**

Purified plasmid DNA was isolated from the transformed *E.coli* cells and subjected to the transformation. *A. tumefaciens* cells were transformed with pFGC5941cEPO vector using electroporation.

4.1.3.20 Transformation of *A. tumefaciens* cells with control expression vector pFGC5941cEPO vector

Competent cells of *A. tumefaciens* were electroporated with 1µg/µl of pFGC5941cEPO vector. Transformed colonies were selected on luria agar plates **(Figure 4.27)** having antibiotics kanamycin (50 mg/L) and rifampicin (50 mg/L). Transformation efficiency was obtained $4.67x10^2$ transformants/ µg of DNA with the 1:6 molecular ratio of the backbone vector and insert vector**.** Transformed colonies were subjected to the PCR screening using Syn7 and cEPO specific primers.

Figure 4.27: Transformed colonies of *A. tumefaciens* with pFGC5941cEPO

4.1.3.21 PCR screening of transformed *Agrobacterium* cells

Selected colonies of *A. tumefaciens* were further subjected to colony PCR to confirm the presence of cEPO and absence of Syn7 intron in vector pFGC5941Syn7cEPO **(Section 3.2.2.9)**. Specific PCR reaction conditions for cEPO and Syn7 (individually) were followed (**Table 3.4 and 3.5)** using transgene specific forwards and reverse primers **(Appendix VI**). Amplified PCR products were analysed on 2 % agarose gel and under UV Gel Documentation unit. Primer designing for Syn7 was mentioned in **Section 4.1.2**. According to this design if the intron is absent in the vector still it amplifies the amplicon of 160 bp. On agarose gel,

amplicon of 160 bp was observed **(Figure 4.28, Lane 1, 2, 3)** which confirmed the absence of Syn7 in the control expression vector. Amplicon of 508 bp was also observed on gel **(Figure 4.28, Lane 9, 10, 11)** which confirmed the presence of cEPO in vector. Size of amplicons was compared with the GeneRuler 50 bp DNA ladder (Fermentas, USA). In the PCR reaction positive and negative both controls were used in the experiment. There was no amplification observed in the negative control **Lane 4** and **8** whereas expected amplicons were observed in positive control **Lane 5** and **7.**

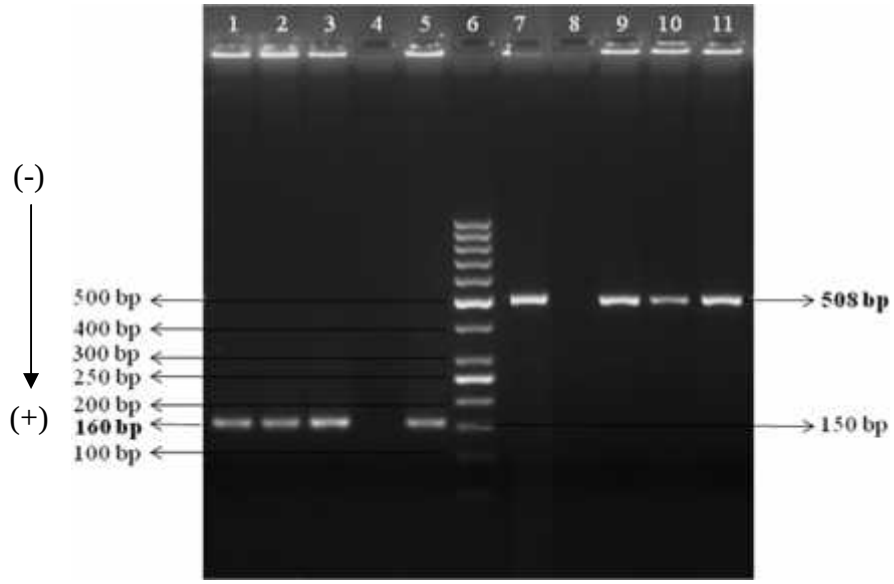


Figure 4.28: Colony PCR confirmation of transformed clones of *A. tumefaciens* with control expression vector pFGC5941cEPO using Syn7 and EPO specific primers.
Lane1-5 (PCR for Syn7 intron) **Lane**1-3 (test samples: transformed colonies), **Lane**4- negative control, **Lane**5- positive control; **Lane**6- 50 bp DNA ladder; **Lane**7-11 (PCR for cEPO) **Lane**7- positive control; **Lane**8- negative control; **Lane**9-11 (test samples: transformed colonies).

Colony PCR screening of the transformed clones of *A. tumefaciens* cells confirmed the successful transformation of *A. tumefaciens* with the control expression vector. Further, these transformed *Agrobacterium* cells were used for the *Agrobacterium*-mediated genetic transformation of *N. tabacum* plant.

In this part of the project, test expression vector and control expression vectors were constructed and their RE and PCR confirmation were done successfully. Final confirmation of both the vectors was performed by the sequencing method.

A. *tumefaciens* cells harboring test expression vector `pFGC5941Syn7cEPO **(Section 4.1.4.2)** and control expression vector pFGC5941cEPO **(Section 4.1.4.10)** were further used for plant transformation studies.

4.2 Plant tissue culture and genetic engineering

4.2.1 Maintenance of *in vitro* culture of *N. tabacum*

In vitro cultures of *N. tabacum* were already maintained as stock culture in our laboratory for various experimentation related to other studies on this plant. For the project, *in vitro* cultures were generated from nodal section of *in vitro* stock cultures of the plants and were maintained throughout the study. Further protocol for shoot regeneration from leaf disc of *in vitro* grown plants was already optimized in the laboratory for different studies (Desai 2009), the same protocol was used in my study.

Murashige and Skoog's (MS) medium supplemented with the combination of plant growth regulators i.e. 2.263 µM Folic acid, 13.95µM kinetin and 5.71µM IAA along with 3% sucrose and 0.9% agar was used for shoot regeneration from nodal section of *in vitro* grown plants This experimental work was performed in laminar air flow hood under aseptic conditions and inoculated flasks were incubated in culture room under standard culture conditions **(Section 3.2.3.3).** Shoot regeneration was observed in the inoculated explants after 1 week of incubation. Regeneration of healthy shoots with 4-5 leaves was observed after 2 weeks of incubation. Fully grown plant with expanded leaves was obtained after 4 weeks **(Figure 4.29 A, B, C).** These healthy green leaves of *in vitro* culture were used as explants in further experimentation.

The MS medium used for the maintenance of *in vitro* culture of *N. tabacum* was named as **MS maintenance medium.**

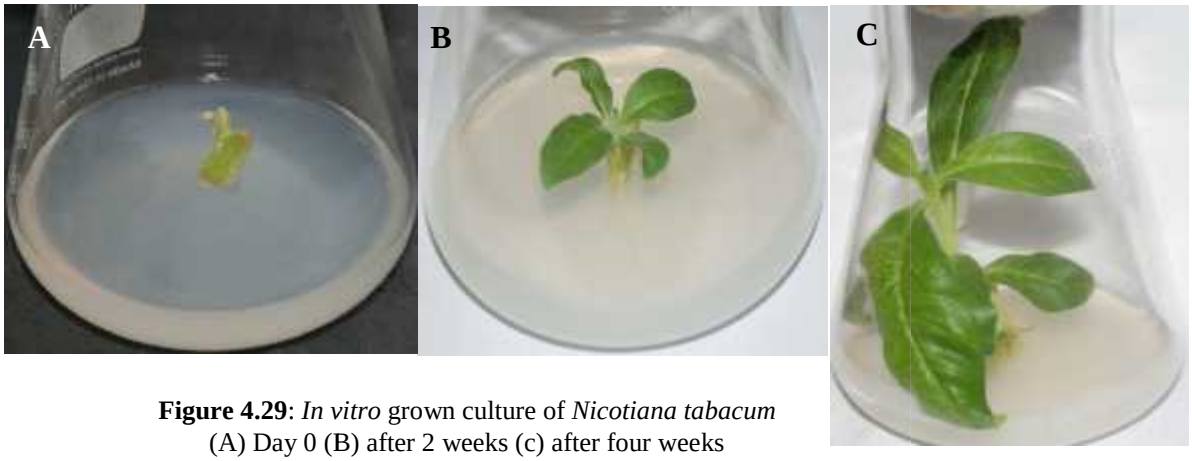
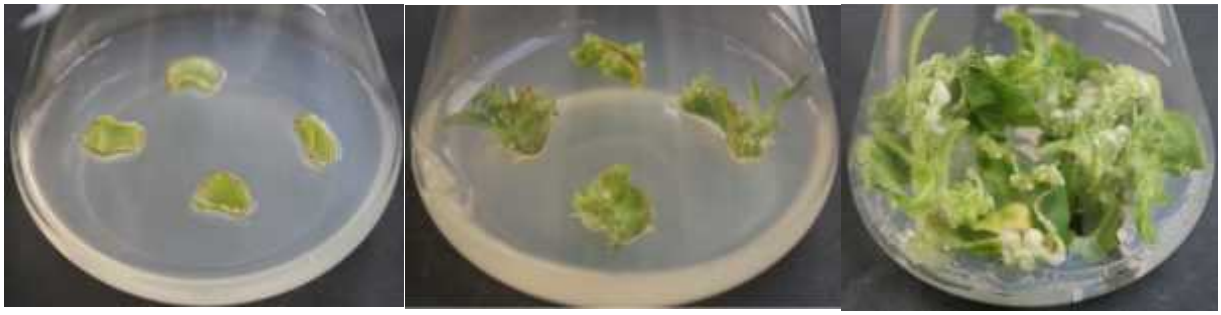


Figure 4.29: *In vitro* grown culture of *Nicotiana tabacum*
(A) Day 0 (B) after 2 weeks (c) after four weeks

4.2.2 Shoot regeneration from leaf disc

Green healthy leaves obtained in previous experiment were used for the shoot regeneration from leaf discs. MS medium supplemented with combination of plant growth regulators i.e. 4.4µM BA and 0.53µM NAA along with 3% sucrose and 0.8% agar **(Table 3.9)** was used for the experiment. Leaf discs were prepared using sterilized cork borer. Four leaf discs were inoculated in one flask and 6 flasks were maintained in the experiment. All the flasks were incubated in the culture room under standard culture conditions **(Section 3.2.3.3)** and periodic observations were recorded. Shoot regeneration signal such swelling and curling in the explants was observed after 3 days of incubation **(Figure 4.30 A).** Microshoots were observed at the wounded or cut part of the leaf discs after 14 days **(Figure 4.30 B**). After 28 days of culture period numerous shoots were regenerated covering entire surface of a leaf disc **(Figure 4.30 C).** The reproducibility of optimized protocol confirms the use of similar protocol in present study for further experiments.

The medium used for shoot regeneration from leaf explants was named as **shoot regeneration medium.**



A **B** **C**

Figure 4.30: *In vitro* shoot regeneration of *Nicotiana tabacum* from leaf discs
(A) Day 3 (B) after 14 days (c) after 28 days

4.2.3 Basta® sensitivity test for selection of transgenic plants

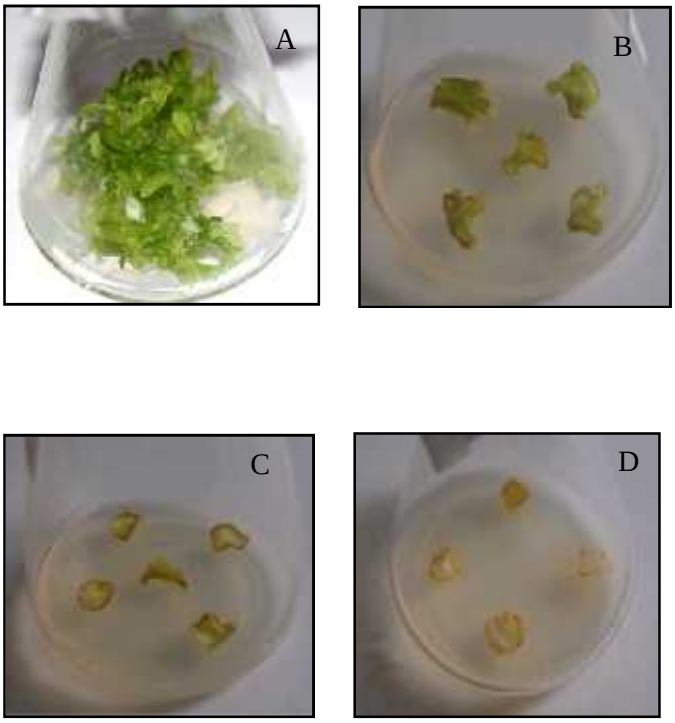
Further experimentation, with *in vitro* maintained stock cultures was related to genetic transformation of the plant using leaf disc as an explants for the study. In plant genetic transformation experiment, it is essential to have selection marker for the selection of transgenic plants. Various plant selection markers such as *Neomycin phosphotransferase, Hygromycin phosphotransferase, Streptomycin phosphotransferase, Chloramphenicol acetyltransferase* genes were used in many previous studies of plant genetic transformation experiment (Miki and McHugh 2004). For the present study Basta® (glufosinate ammonium) was used for selection of putative transformants as *bar* gene was present in the vectors to be used for the genetic transformation studies **(Section 4.1).** The vectors in this study were pFGC5941Syn7cEPO and pFGC5941cEPO **(Section 4.1.4.2 and 4.1.4.10).**

Bar gene confers the resistance against Basta®, enabling transformants to grow in Basta® supplemented medium. Basta® sensitivity assay was carried out for the determination of the optimum concentration of Basta® which is required for the selection of transgenic plants. Basta® is a herbicide and it is widely used as a selection agent for transgenic plants carrying *bar* gene as a selection marker (Miki and McHugh 2004). Basta® contains L-isomer of phosphinothricin (PPT) (glufosinate ammonium) as an active ingredient. This L-isomer of PPT is an analogue of L-glutamic acid and acts as a competitive inhibitor of glutamine synthetase (GS). Glutamine sythetase catalyses conversion of ammonia into glutamic acid in plants. However, inhibition of glutamine synthetase leads to the accumulation of toxic ammonia which causes plant cell death (OECD, 1999). *Bar* gene confers resistance against Basta® by deactivating PPT by its acetylation and stop ammonia accumulation in plant cells (De Block et al. 1987).

In this experiment various concentrations of Basta® were added in the MS shoot regeneration medium after sterilization under aseptic conditions. Fresh leaf discs of *N. tabacum* were placed on medium containing 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mg/L concentration of Basta® and incubated under standard culture conditions for 28 days. Flasks containing MS shoot regeration medium without Basta® were also inoculated with leaf discs and were used as control for the experiment **(Figure 4.31 A).** After 28 days it was observed that lower concentrations (<1 mg/L) were not toxic to the explants as shoot regeneration was observed in explants **(Figure 4.31 B)** whereas higher concentration (more than 1 mg/L) resulted in

necrosis and chlorosis in explants **(Figure 4.31 D).** Therefore, 1 mg/L concentration of Basta® was found to be optimum for the selection of transformed plants on MS selection media **(Figure 4.31 C Table 4.1).** In a reported study, for the selection of transgenic *N. tabacum*, 2-10 mg/L Basta® concentration was used and concentration of 2 or 5 mg/L of Basta® was observed to inhibit the growth or regeneration of shoots from leaf discs on selection medium (Viegas PM and Notani NK 1993). In present study, 1 mg/L concentration was found optimum for the selection of transgenic plants. Basta® concentration higher than 1 mg/L were showing the toxic response by causing cell death in the leaf discs tissues.

Figure 4.1 Effect of various concentrations of Basta® on leaf discs



Sr. No.	Concentration (mg/L)	Result
1.	0.25	Growth
2.	0.5	Growth
3.	1	Inhibited growth
4	2	Necrosis, chlorosis
5.	3	Necrosis, chlorosis
6.	4	Necrosis, chlorosis
7.	5	Necrosis, chlorosis
8.	6	Necrosis, chlorosis

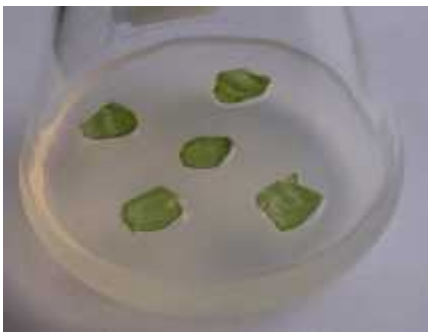
Figure 4.31: Basta® sensitivity test with *N. tabacum* leaves
(A) Control (Without Basta®); (B) 0.5 mg/L; (C) 1.0mg/L;
(D) 2 mg/L

This optimum concentration of Basta® (1mg/L) which was selected from the Basta® sensitivity test was used for the selection of the transformed plants of *N. tabacum* in genetic engineering studies of the project.

4.2.4 Genetic transformation of *N. tabacum*

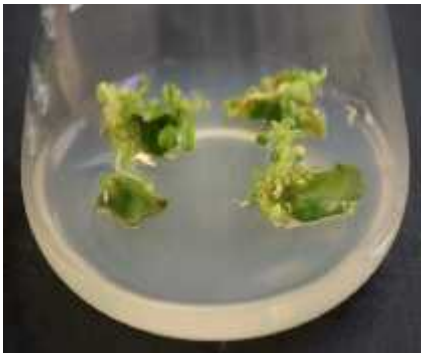
Agrobacterium-mediated genetic transformation is one of most easy, compatible and robust technique for the genetic manipulation or transformation of the dicot plants (Tzfira and Citovsky 2006). In present study leaf discs of *N. tabacum* were transformed with *Agrobacterium tumefaciens* strain LBA4404. Following the methodology explained in **Section 3.2.3.6b**.

Fresh green and young leaves of *N. tabacum* were used to prepare leaf discs. MS shoot regeneration medium was used to pre-condition the explants before transformation. After 48 h of pre-conditioning, leaf explants were infected with *A. tumefaciens* culture harboring test expression (pFGC5941Syn7cEPO) and control expression vector (pFGC5941cEPO) constructed during molecular biology experiments. After infection with *Agrobacterium* the explants were blotted on sterilized blotting paper to remove excess medium and placed back to the pre-conditioning medium for co-cultivation for 48 h. After 48 h, explants were transferred on MS shoot regeneration medium supplemented with cefotaxime antibiotic 250 mg/L and incubated for 12 days to prevent the excessive growth of the *Agrobacterium* cells. After 12 days, growth of shoots was observed on MS regeneration medium containing cefotaxime. MS medium supplemented with the Basta® (1 mg/L) and cefotaxime (250 mg/L) was prepared for the selection of putative transformants. All the explants were transferred on the selection medium and incubated for 28 days. Thereafter, transformed plants were subcultured every 28 days intervals on the same medium for their growth. Growth of the putative transformants on selection medium indicates the presence or integration of *bar* gene in the plant cells. *Bar* gene confers resistance against Basta® and allow the transgenic plants to grow on MS selection medium containing Basta®. On the basis of survival of leaf explants on selection medium the transformation efficiency was calculated **(Figure 4.32 and 4.33)** 86.67± 0.58% for plants transformed with pFGC5941cEPO vector (control expression vector) and 73.33±2.08% for plants transformed with pFGC5941Syn7cEPO vector (test expression vector). With these sets of experiment non-infected explants were also inoculated in the MS medium to observe the normal growth of regenerated plants from the leaf discs **(Figure 4.34).**



A

B



C

D

E

Figure 4.32: Transformed plants of *N. tabacum* with test expression vector pFGC5941Syn7cEPO; (A) Preconditioning leaf explants; (B) Co- cultivated leaf explants with *Agrobacterium* culture; (C) Leaf explants after 10 days on cefotaxime containing medium; (D) Putative transformants on selection medium after 28 days; (E) Transformed plants after 10 weeks.

A

B

C

D

E

Figure 4.33: Transformed plants of *N. tabacum* with control expression vector pFGC5941cEPO; (A) Preconditioning leaf explants; (B) Co- cultivated leaf explants with Agrobacterium culture; (C) Leaf explants after 10 days on cefotaxime containing medium; (D) Putative transformants on selection medium after 28 days; (E) Transformed plants after 10 weeks.

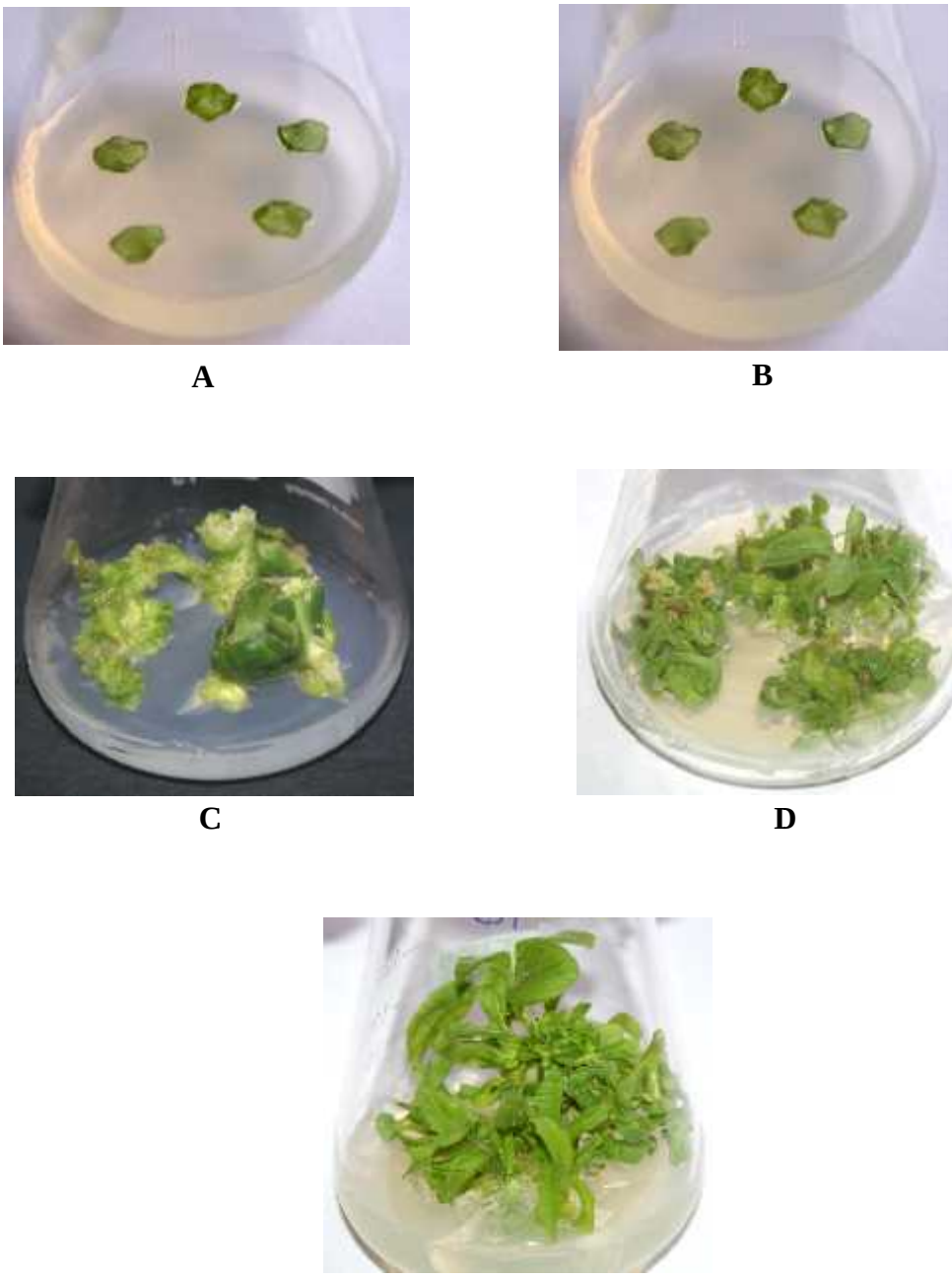


Figure 4.34: Non transformed plants of *N. tabacum* (A) leaf explants at day 0 (B) After two days (C) Leaf explants after 10 days (D) after 28 days (E) after 10 weeks.

Transgenic plants grew efficiently under *in vitro* conditions and further, growth of the transgenic plants was also analyzed in *in vivo* conditions. So, *in vitro* grown transgenic plants were subjected to the rooting medium for induction of roots and then for hardening.

4.2.5 Rooting of the transformed plants

After 3 months of growth of the *in vitro* shoot culture, elongated shoots of *in vitro* grown transformed and non-transformed plants were excised and placed on the rooting medium containing the combination of 2.263 µM Folic acid, 13.95µM kinetin and 5.71µM IAA along with 3% sucrose and 0.8 % agar **(Section 3.2.3.6c; Table 3.8)** and incubated in culture room under standard culture conditions. After 9 days initiation of roots was observed which then converted in to an immense cluster after 28 days of growth **(Figure 4.35).** Growth and expansion of leaves were also observed.

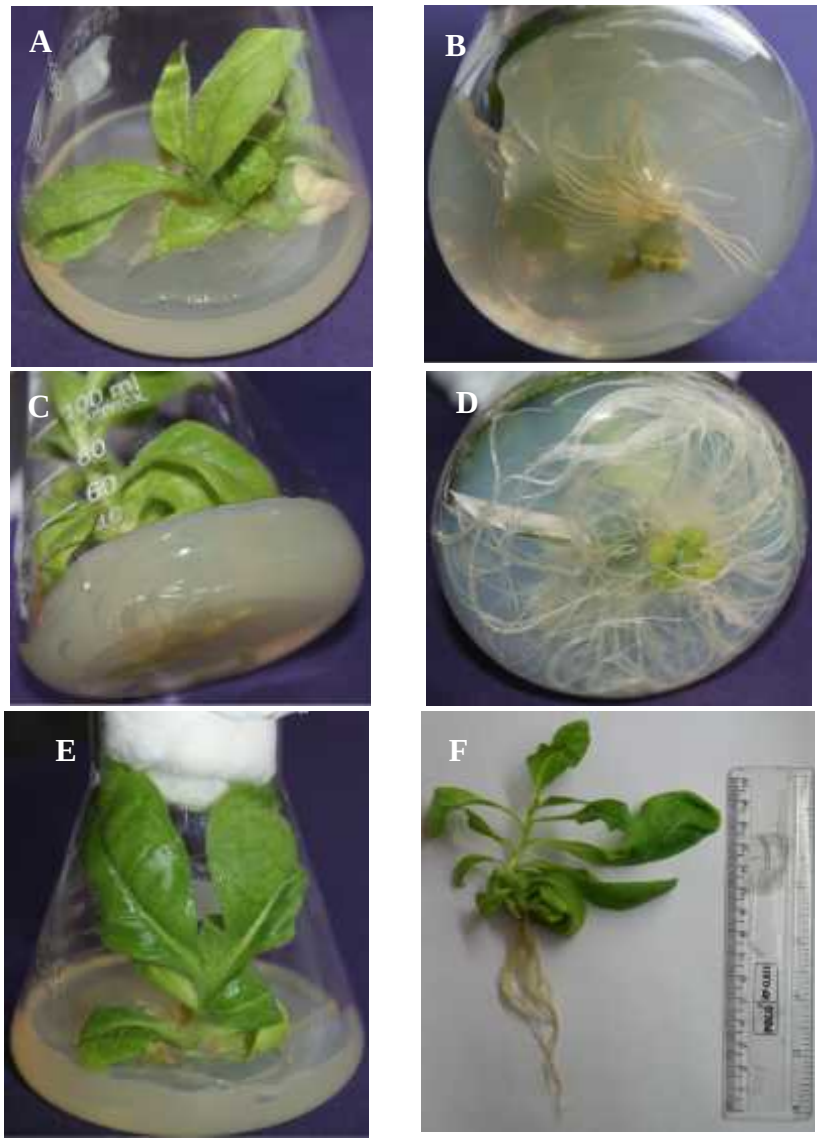
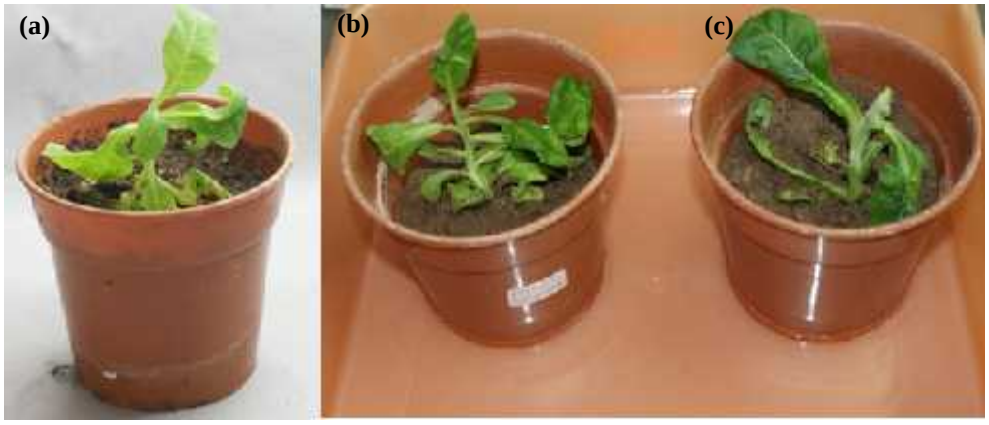


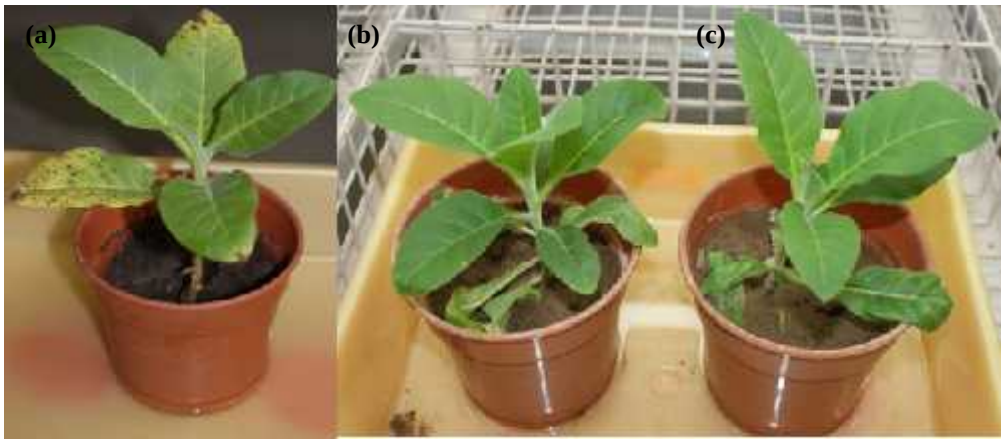
Figure 4.35: A, B, C *In vitro* grown plant in rooting medium at day 15; **D, E, F** *In vitro* grown plants in rooting medium with clusters of roots at day 30.

4.2.6 Hardening of *In vitro* grown transgenic plants of *N. tabacum*

After 28 days, *in vitro* plantlets (shoot with roots) were subjected to hardening in sterile soil:sand mixture (1:1). *In vitro* grown rooted plantlets were taken out of the flasks and root part of the plant was washed with sterile water to remove the residual agar and this plantlet was transferred in to the pot containing sterile soil and sand mixture (1:1). Sterile water was poured in the pot to nourish the plants **Section 3.2.3.6d**. Plants were placed in the culture room under standard culture conditions. Plants were nourished every alternate day with water and liquid basal MS-medium (50:50). The plants remained green and healthy and new leaves started appearing after a week **(Figure 4.36).** The transformed and non-transformed plants grew under *in vivo* conditions were healthy.



(i)



(ii)

Figure 4.36: Hardening of *in vitro* regenerated plantlets at (i) day 0; (ii) after day 28
(i): (a) Non-transformed plant (b) transformed plants with test expression vector (c) transformed plants with control expression vector
(ii): (a) Non-transformed plant (b) transformed plants with test expression vector (c) transformed plants with control expression vector

4.3 Analysis of transgenics

Simultaneously, with rooting and hardening experiments, *in vitro* grown transgenic and non-transgenic plants were subjected to various experiments, regarding confirming the transgene integration in the genomic DNA of *N. tabacum* and expression of erythropoietin in transgenic plants.

4.3.1 Analysis of transgene integration

Total genomic DNA was isolated for the analysis of integrated transgenes in to genomic DNA of *N. tabacum* and subjected to the PCR and southern blot analysis.

Integration of all the transgenes such as cEPO, bar, syn7 in to genomic DNA of *Nicotiana tabacum* was confirmed by polymerase chain reaction using transgene specific primers. Ribosomal protein L25 was selected as endogenous control gene as its expression does not fluctuate much under stress conditions. Southern blot analysis was also performed for further confirmation of the bar gene integration in to the genomic DNA of *N. tabacum.*

4.3.1.1 Isolation of genomic DNA from putative transformants of *N. tabacum*

Genomic DNA was isolated from 5 randomly selected plants of each of the 3 category i.e.

(i) Non-transformed plants (ii) Plants transformed with control expression vector (pFGC5941cEPO), (iii) Plants transformed with test expression vector (pFGC5941Syn7cEPO) using CTAB method (**Section 3.2.4.1).** CTAB method is one of the well known and established methods for the isolation of genomic DNA from plants. This method is widely used in PCR, RAPD and AFLP fingerprinting techniques for the easy and fast isolation of genomic DNA from various plant samples (Stewart and Via 1993; Stewart 1997). Isolated genomic DNA was quantitatively analysed by spectrophotometry. The 260/280 ratio of genomic DNA was observed in the range of 1.78- 1.85 and concentration of was observed in range of 0.9 µg/µl- 1.3 µg/µl. Genomic DNA was further subjected to gel electrophoresis for the qualitative analysis. 1 µg of each DNA sample was run on 0.8% agarose gel and observed under gel documentation unit **(Figure 4.37).**

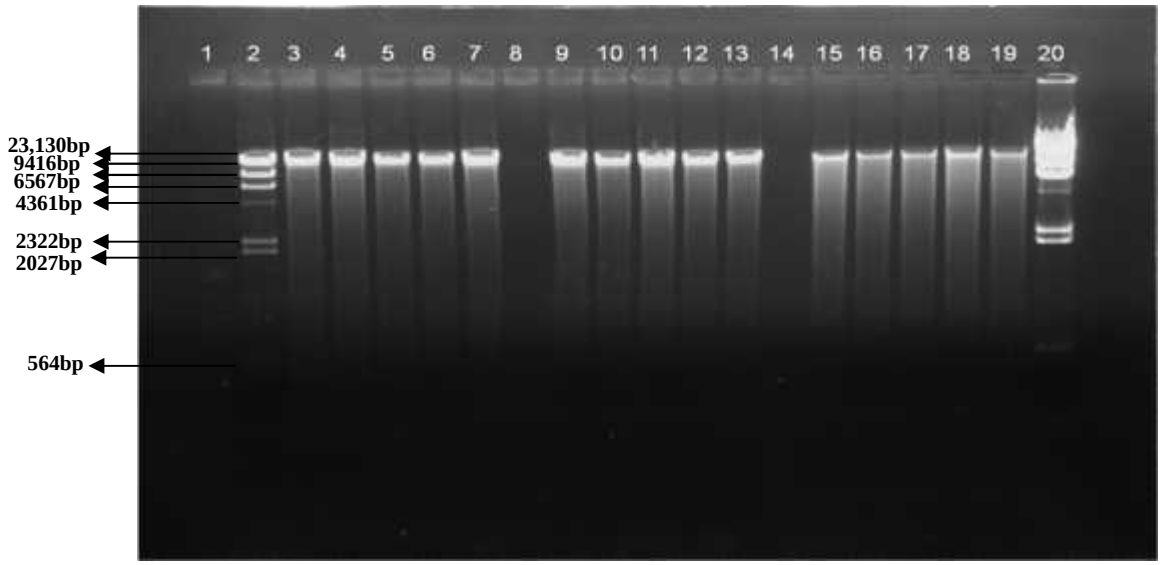


Figure 4.37: Isolated genomic DNA from; non-transformed plants (3-7); transformed plants with pFGC5941Syn7cEPO (9-13); transformed plants with pFGC5941cEPO (15-19); Lambda DNA marker (20); (lane 1, 8 and 14 blank).

Lambda DNA marker *Hind*III digested (larger fragment of 23 kb) was also run with genomic DNA of *N. tabacum* to assures the presence of high molecular weight genomic DNA. Isolated genomic DNA of transformed and non-transformed plants of *N. tabacum* was further used in PCR and southern blot analysis for the confirmation of transgene integration in genomic DNA of the plants.

4.3.1.2 Multiplex PCR confirmation of transgenics

Multiplex PCR is used to amplify simultaneously, the two or more genes using same amplifying parameters and in a single reaction mixture. Nowadays, it is widely gaining interest in the field of clinical research and laboratory research (Markoulatos et al. 2002).

In the present study, genomic DNA of transformed plants based on the Basta® selection and non-transformed plants were isolated and subjected to multiplex PCR **(Section 3.2.4.2, Table 3.11)** using transgenes specific primers **(Appendix VI).**

a) Plant transformed with test vector (pFGC5941Syn7cEPO)

As the *N. tabacum* was transformed with pFGC5941Syn7cEPO vector. So, the presence of all the expected transgenes should be there in the genomic DNA of *N. tabacum*. To confirm the integration of transgenes (cEPO, *bar*, Syn7) in the genomic DNA of *N. tabacum* multiplex

polymerase chain reaction technique was carried out using transgene specific primers. For the confirmation of the genomic DNA from the plant origin, endogenous gene (Ribosomal Protein L25) was also amplified with the each transgene. PCR amplified product of all the genes were analyzed on 2% agarose gel. Intense and amplified amplicons of integrated transgenes (508 bp amplicon of cEPO, 246 bp amplicon of *bar* gene, 394 bp amplicon of Syn7) were observed on agarose gel with the amplicon of endogenous gene (160 bp). In the negative control reaction mixture and all the primers were put with the PCR reaction mixture without DNA template. No amplification was observed in the negative control reaction. Size of all the amplicons was compared with the 50 bp DNA ladder **(Figure 4.38).**

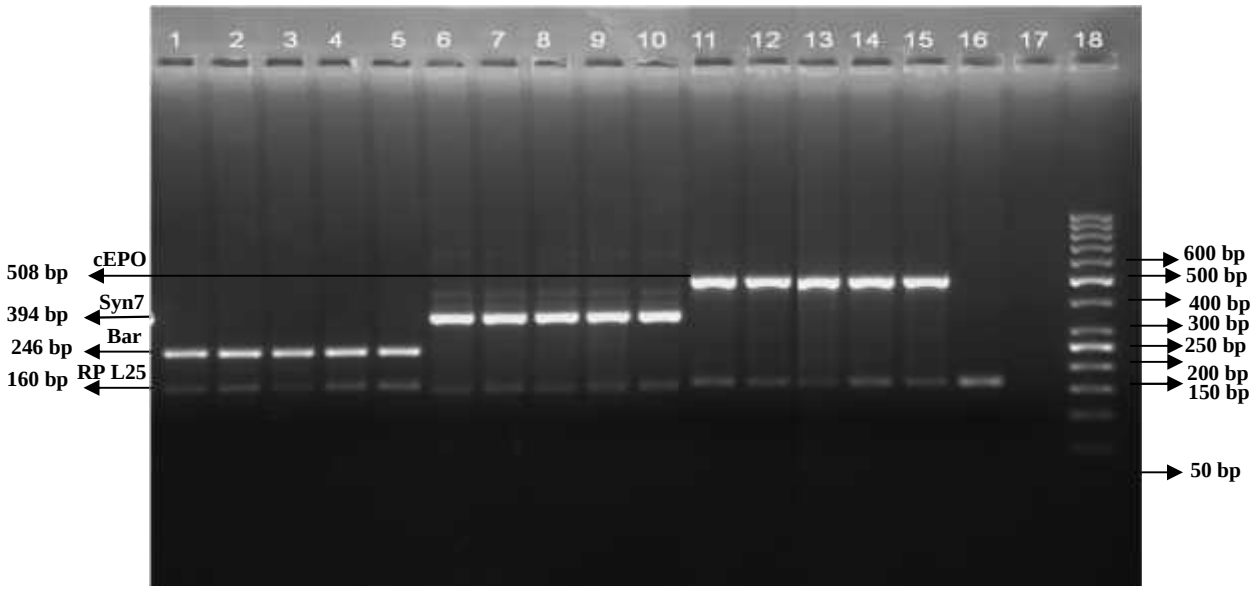


Figure 4.38: PCR confirmation of putative transformants transformed with test expression vector pFGC5941Syn7cEPO vector: Lane 1-5 amplification of bar gene and endogenous gene RP L25; Lane 6-10 syn7 and RP L25; Lane 11-15 cEPO and RP L25; Lane 16 non transformed plant; Lane 17 negative control; Lane 18 Gene Ruler 50bp gene ladder.

b) Plants transformed with control vector (pFGC5941cEPO)

Transformed plants of *N. tabacum* with pFGC5941cEPO vector should have the integrated transgene such as EPO and *bar.* Multiplex PCR amplified products on 2% gel showed amplicon size of 508 bp of epo, 246 bp of bar gene, 160 bp of Syn7 and 160 bp fragments of L25 genes under gel documentation unit. Primers of syn7 intron were designed as described in **Section 4.1.2**. Therefore the absence of intron was also confirmed by presence of 160 bp

amplicon as primers were designed by covering the flanking region of Syn7 intron. The amplicon size of the endogenous control gene and the syn7 flanking region was same (160 bp). The presence of highly intense amplicon on agarose gel represents the presence of both amplicon (L25 gene and absence of Syn7 gene) in the same lane. No amplification was observed in the negative control reaction and amplification of only RP L25 gene was observed in non-transformed plants. Size of all the amplicon was compared with the 50 bp DNA ladder **(Figure 4.39).**

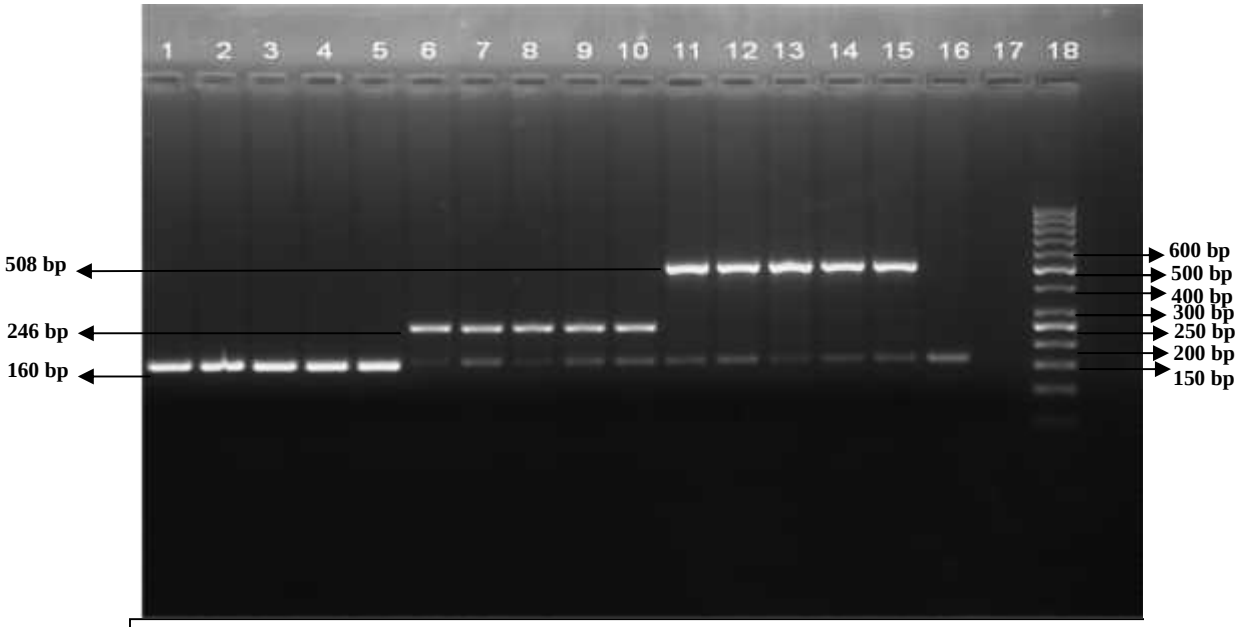


Figure 4.39: PCR confirmation of non transformed and transformed plants with pFGC5941cEPO vector: Lane 1-5 amplification of Syn 7 and endogenous gene RP L25; Lane 6-10 bar gene and RP L25; Lane 11-15 cEPO and RP L25 gene; Lane 16 non transformed plant; Lane 17 negative control; Lane 18 Gene Ruler 50bp gene ladder.

Amplification of the endogenous control gene with each transgene revealed the preliminary confirmation of the integration of transgenes in genomic DNA of *N. tabacum*.

4.3.1.3 Southern blot analysis

Southern blot technique is applied to detect the presence of specific gene or DNA sequence in the DNA sample. This technique involves the fragmentation of the genomic DNA in to small fragments using restriction endonuclease enzymes, transfer and blotting of the fragmented DNA samples from agarose gel to membrane and finally detection of the specific DNA or gene using hybridization probe (Southern 1975).

In the present study nonradioactive DIG labeled probes were used to analyze the integration of *bar* gene in the genomic DNA of the *N. tabacum* using DIG (digoxigenin) DNA labeling and detection kit. Protocol was followed as mentioned in section 3.2.4.3. Digoxigenin is the non-radioactive probe and it is used to label the nuclei acid. DIG-labeled non-radioactive probes has been used in many studies to detect the transgene integration in plant genome such as rice, potato, sugar beet, maize, and wheat (Dietzgen et al. 1999).

In present study, isolated genomic DNA from transformed and non-transformed plants which have been found positive in PCR analysis **(Section 4.2.1.1)** were used in southern blot analysis. Genomic DNA (25µg) was completely digested with *Eco*RI restriction enzyme and run on 1% agarose gel. Current at 1-5 V/cm was applied for 90 min. After gel electrophoresis, the gel was placed in the denaturing solution for 10 min and then in neutralising solution for 30 min. Then it was placed on the membrane for the transfer of the whole digested genomic DNA on membrane by capillary action. Further, membrane was hybridized with probe and subjected to the colour development reaction. Resulting, purple colour bands on the membrane in **Lane 3 and 4 (Figure 4. 40)** which revealed the presence of *bar* gene in genomic DNA of transformed plants of *N. tabacum* and absence of purple band was observed in genomic DNA of non-transformed plants of *N. tabacum* present in **Lane 1; Figure 4.40.** PCR amplified product of *bar* gene from the genomic DNA of transformed plant of *N. tabacum* was used as a positive control which also resulted in purple colour band on membrane**.** In the present study, presence of single purple band on the membrane represents the integration of single copy of *bar* transgene. The purple band was appeared on the membrane due to the hybridization of the DIG-labeled bar specific probe with the integrated *bar* transgene present in the genomic DNA of *N. tabacum*.

In another reported study, the sensitivity of DIG (Digoxigenin) DNA labeling kit was analysed by the detection of a housekeeping gene (elf-1) in the genome of *Arabidopsis*

thaliana plant under different environmental conditions and it was found that digoxigenin is very efficient and sensitive to detect the presence of gene in the genomic DNA of *A. thaliana.* Even the single copy of a gene in the plant genome can be detected (Hart and Basu 2009). Results obtained in the present study are also in accordance with results obtained in previous studies as the transgene was efficiently detected by DIG (Digoxigenin) DNA labeling kit.

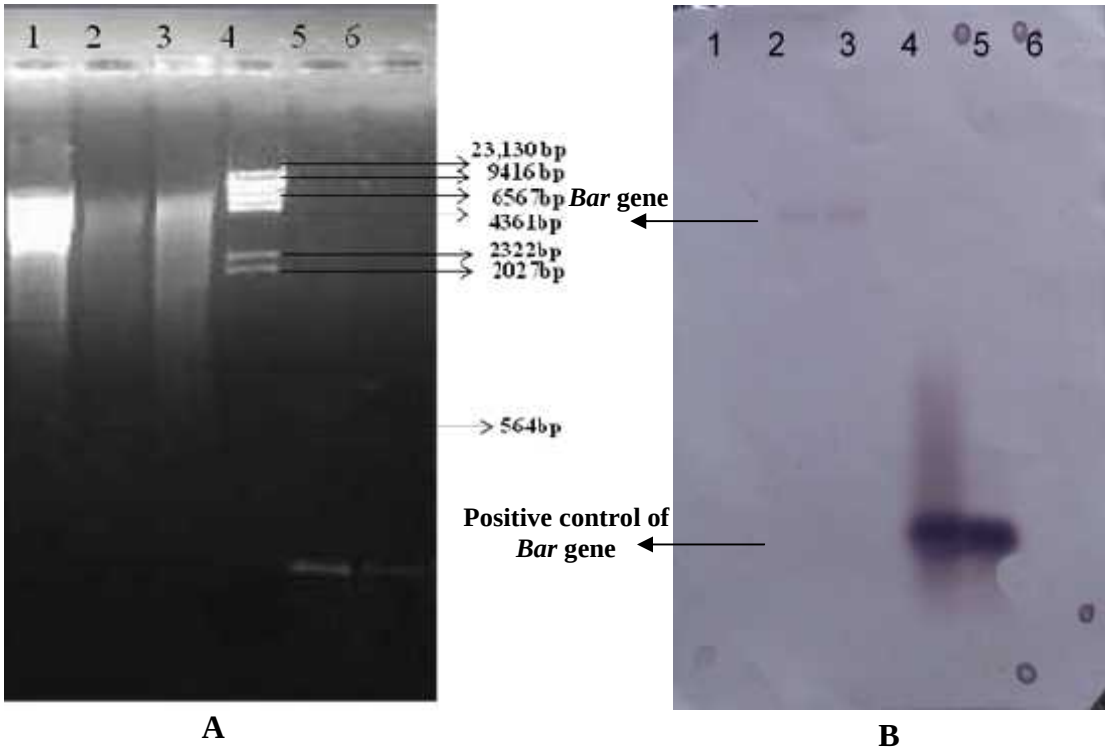


Figure: **4.40** Southern blotting of integrated *Bar* gene in Transformed plant of *N. tabacum*:
(A) Agarose gel picture: Lane 1 Genomic DNA of non-transformed plant; Lane 2 Genomic DNA of transformed plant; Lane 3 Genomic DNA of transformed plant; Lane 4 λ DNA Ladder; Lane 5 & Lane 6 PCR product of *Bar* gene.
(B) On membrane: Lane 1 No colour band; Lane 2 & 3 colour band of bar gene in transformed plant, Lane 4. Lambda DNA ladder; Lane 5 & Lane 6 colour bands of positive control (PCR product of *Bar*

These all results confirmed the successful integration of the transgenes (*bar*) in the genomic DNA of transformed plants of *N. tabacum.*

Further, genetic modification induced stress in transformed and non-transformed plants were studied using HPLC techniques by the analysis of nicotine alkaloid content in N. tabacum plant.

4.3.2 Estimation of Nicotine content by HPLC

N. tabacum contains four types of major alkaloids i.e. anatabine, anabasine, nicotine and nornicotine. Nicotine accounts for 90-95% of the plants' total alkaloid content (Hashimoto and Yamada 1994). For the present study, nicotine content in non-transformed and transformed plants (with pFGC5941Syn7cEPO and pFGC5941cEPO) was analyzed using High Performance Liquid Chromatography (HPLC) technique.

Nicotine standard was used for method optimization. 100 µg/ml working concentration of nicotine standard was prepared in methanol. 500 µl of the standard was taken in sample tube and 10 µl of standard sample was injected in the column. K_2HPO_4 buffer of pH 5.8 was used and found optimum to resolve the peak under 11 mins of retention time. Intraday and interday method validation for nicotine was performed as mentioned in **Section 3.2.4.4a.** Calibration curve was constructed using linear regression of peak area versus concentration of the standards. Ranging 1 µg/ml to 100 µg/ml concentration of standard nicotine was used to prepare calibration curve which resulted in 0.999 value of Regression (R^2) coefficient. The amount of nicotin in the unknown samples was calculated from the calibration curve. The obtained Regression (R^2) 0.999 for the calibration curve indicates the gradual increase in area value with the increased concentration of nicotine **(Figure 4.41a, Table 4.3).**

Further, 4 biological replicates (samples from different plants) were taken of each transformed and non-transformed samples with 3 technical replicates (samples from same plants) of each biological samples for HPLC analysis. Sample extract of non-transformed and transformed plants of *N. tabacum* were prepared as mention in **section 3.2.4.4b** and injected (10 µl) in the column. Concentration of unknown samples was determined by the area obtained from the each injection.

As per the result of HPLC analysis, nicotine content was found to be significantly higher ($p<0.0001$) in transformed plants as compared to non-transformed plants of *N. tabacum* **(Figure 4.41c, Table 4.4)**.

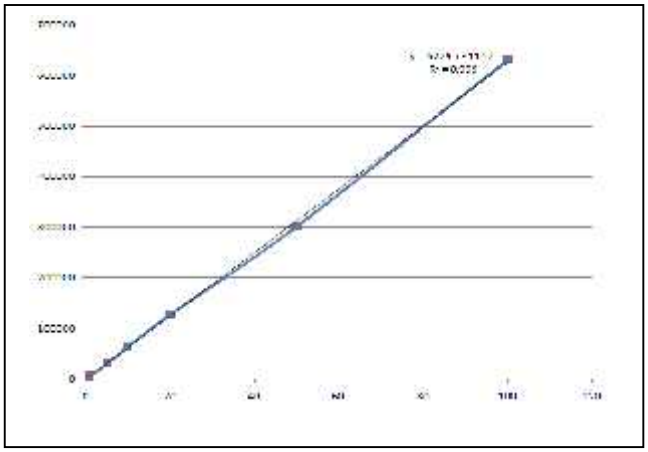


Figure 4.41 a: Calibration curve of Nicotine standard

Table 4.2: Concentration of standard nicotine

S. No.	**Nicotine** µg/ml
1	1.00 µg
2	5.00 µg
3	10.0 µg
4	20.0 µg
5	50.0 µg
6	100 µg

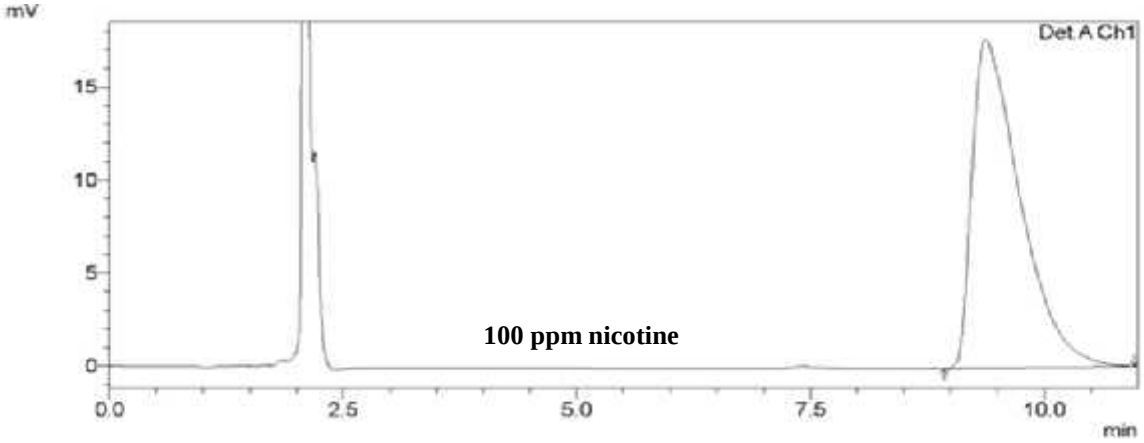


Figure 4.40b: HPLC chromatogram of nicotine standard

Table 4.3: Nicotine content in transformed and non-ransformed plants of *N. tabacum*

S. No.	***N. tabacum***	**Concentration of nicotine content (µg/ml)**
1	Non-transformed plants (N)	7.932
2	Transformed plants with pFGC5941cEPO	15.919
3	Transformed plants with pFGC5941Syn7cEPO	16.915

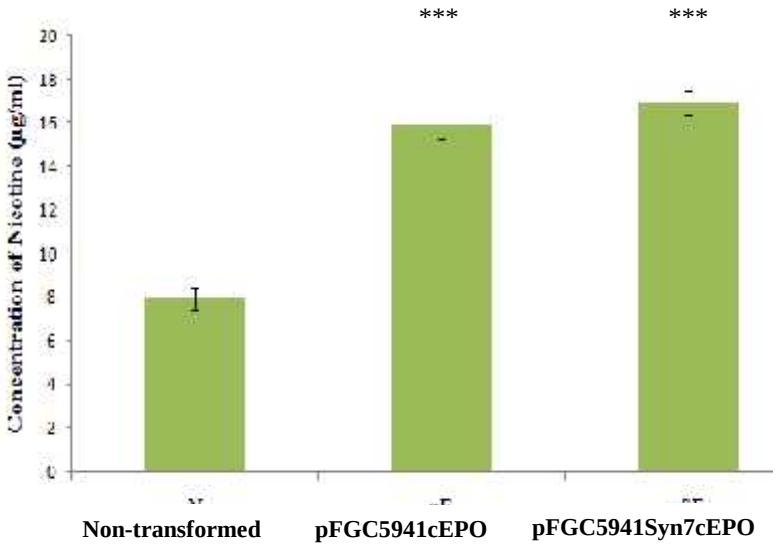


Figure 4.41c: HPLC analysis for the comparative quantitative analysis of nicotine in non-transformed plant and transformed with control and test expression vectors extract of *N. tabacum.*
pFGC5941cEPO (control expression vector); pFGC5941Syn7cEPO (test expression vector) Dunnett's test was applied for the comparative analysis of nicotine content. Error bar indicates standard deviation; (***), ($p<0.0001$) indicates the level of significance.

Arruda et al. in 2013 revealed that effect of genetic manipulation, can be considered as a stress which can lead to the metabolic burden. This metabolic burden can result in differential expression of genes (Arruda et al. 2013). Similarly, in the present study also drastic difference was observed in the level of nicotine content in the transformed plants as compared to non-transformed plants which indicates the induction of metabolic stress in transformed plants which is evinced by the significant high levels of nicotine content. Absence of metabolic burden stress in non-transformed plant is clearly indicated by the low level of nicotine.

The present study discloses that plant defence system may get activated due to the genetic manipulation. Resulting, in high secretion of secondary metabolites by plants against the induced stress which is clearly evident in the present study in the form of increased nicotine content in transgenic plants as compared to non-transgenic plants of *N. tabacum*.

So, this experiment is an indirect confirmation of the transformed plants, as metabolic stress is found higher in transformed plants.

4.3.3 Analysis of expression of erythropoietin at transcriptional (mRNA) level

Expression of erythropoietin in the transformed plants of *N. tabacum* with test expression vector (containing Syn7 intron and EPO) and control expression vectors (containing EPO only) was confirmed at transcriptional level. For the mRNA expression analysis total RNA was isolated, its integrity was checked and used for the qualitative and quantitative analysis of mRNA expression.

4.3.3.1 Total RNA isolation

RNeasy kit (Qiagen) was used for the isolation of total RNA from plant as mentioned in **Section 3.2.4.5a**. Total RNA was quantified by spectrophotometry. The ratio 260/280 for total RNA was observed around 1.91- 2.23 and concentration was found around 70 ng/µl - 120 ng/µl. 1µg of isolated RNA was run on 1% agarose gel and observed under UV illuminator **(Figure 4.42).**

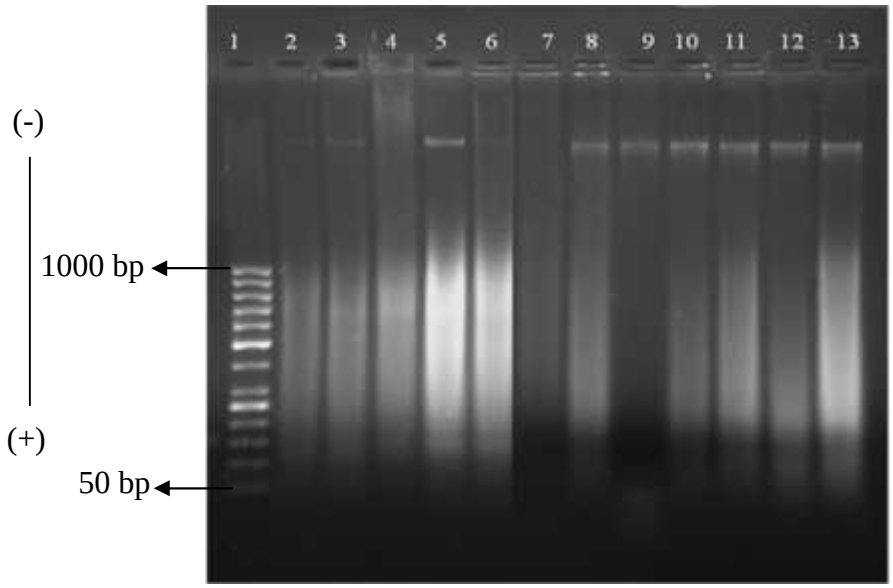


Figure 4.42: Total isolated RNA from non-transformed and transformed plants of *N. tabacum*; Lane 1 Gene Ruler 50 bp ladder; Lane 2-5 RNA from transformed plant of *N. tabacum* with pFGC5941Syn7cEPO; Lane 6-9 RNA from transformed plant of *N. tabacum* with pFGC5941cEPO; Lane 10-13 RNA from non-transformed plant.

4.3.3.2 Formaldehyde agarose (FA) gel electrophoresis

Formaldehyde agarose gel electrophoresis is used to check the size and integrity of RNA preparations (protocol mentioned in **Section 3.2.4.5b**). RNA can form many different secondary or tertiary structures which can affect its separation in an electrical field, and it cannot be maintained in a denatured state. Formaldehyde was used to denature the RNA. After electrophoresis, the RNA was visualized by staining with ethidium bromide **(Figure 4.43).**

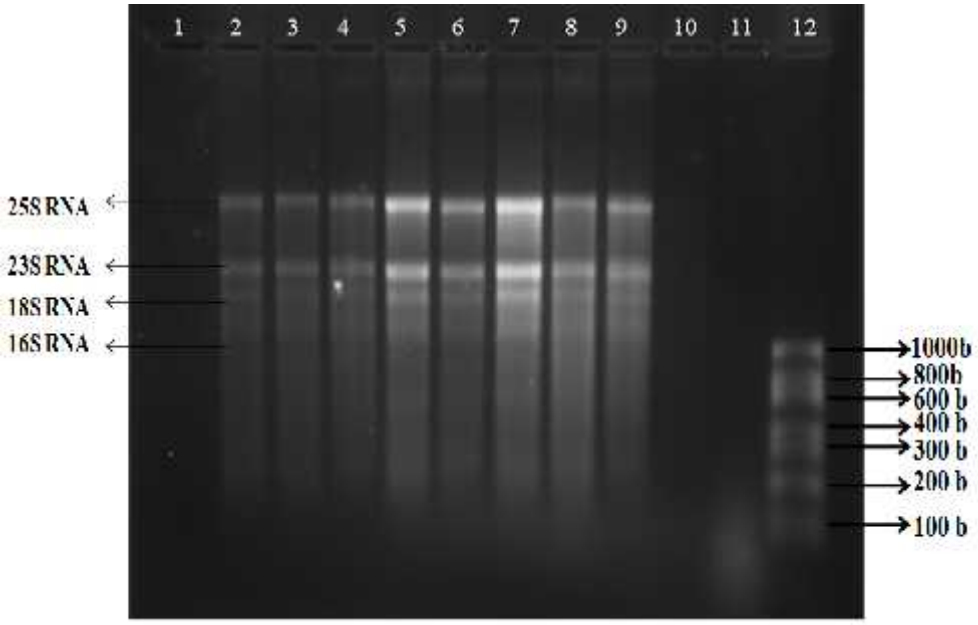


Figure 4.43: Formaldehyde Agarose Gel Electrophoresis for the isolated RNA samples from non-transformed and transformed plants of *N. tabacum*; Lane 2-4 RNA samples from transformed plants with pFGC59415941Syn7cEPO; Lane 5-7 RNA samples from transformed plants with pFGC59415941cEPO; Lane 8-9 RNA from non-transformed plant; Lane 12 low range RiboRuler.

After the analysis of the RNA quality and its integrity, it was used for the cDNA preparation, The cDNA thus obtained was used for the qualitative and quantitative analysis of mRNA expression of erythropoietin in transformed plants of *N. tabacum* with test expression vector and control expression vector.

4.3.3.3 cDNA Preparation

cDNA was prepared from the total RNA isolated from non-transformed and transformed plants of *N. tabacum* using 5X Iscript supermix under the specific PCR conditions as mentioned in **Section 3.2.4.6**.

Prepared cDNA was used for qualitative analysis and further for quantitative analysis of mRNA expression of erythropoietin using real time PCR in *N. tabacum* plants transformed with test expression vector (with intron construct) and control expression vector (without intron construct).

4.3.3.4 Qualitative mRNA expression analysis

cDNA (5 µl) was used for the PCR amplification of EPO (transgene) and ribosomal protein L25 genes (endogenous control gene) using genes specific primers. Similar PCR reaction and conditions were used for the amplification of cDNA which were used for the PCR amplification of the genomic DNA of putative transformants as mentioned in **Section 3.2.4.7**. PCR products were run on 2% agarose gel and visualised under gel documentation unit. The EPO amplicon of 508 bp was not observed in the **Lanes 4-6** as they all were loaded with PCR product amplified with cDNA obtained from RNA samples of non-transformed plants. Amplicon of 508 bp of EPO was observed in **Lanes 8-10** (transformed with pFGC5941Syn7cEPO) and **Lanes 11-13** (transformed with pFGC5941cEPO) which revealed the expression of EPO in both the samples. Endogenous control gene RP L25 was also amplified from each samples of cDNA (prepared from transformed and non-transformed plants' RNA samples). Good difference was observed in the band intensity of the PCR amplified cDNA samples of the plants transformed with pFGC5941Syn7cEPO (with intron construct) and plants transformed with pFGC5941cEPO vectors (without intron construct) **(Figure 4.44).** Band intensity PCR amplified endogenous control gene was found almost same in all the samples. Size of all the amplicons was compared with the 50 bp DNA ladder.

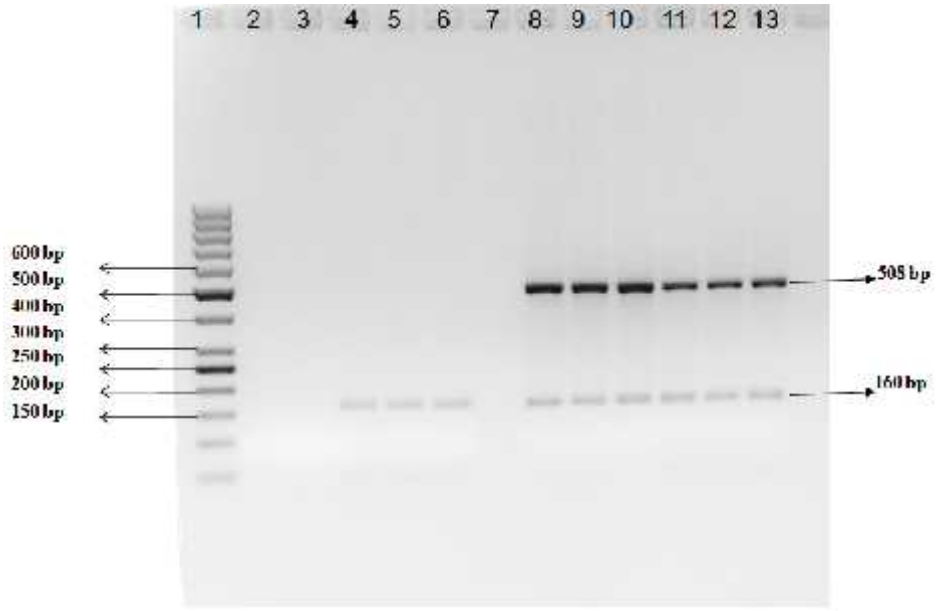


Figure 4.44: Qualitative analysis of mRNA expression of cEPO
Lane 1 50 bp DNA ruler ladder; Lane 2-3 Negative control; Lane 4-6 non transformed plants; Lane 8-10 transformed plants with pFGC5941Syn7cEPO; Lane 11-13 transformed plants with pFGC5941cEPO

In qualitative mRNA expression analysis, the expression of erythropoietin was observed higher in *N. tabacum* plants transformed with expression vector having intron construct (test expression vector) as compare to intron less construct (control expression vector). Afterwards quantitative mRNA expression analysis was also performed to analyse the expression of erythropoietin in plants using real time PCR.

4.3.3.5 Quantitative mRNA expression analysis of cEPO and endogenous gene RP L25 using Real Time PCR method

Real time PCR is the method used for the mRNA expression analysis due to the ease and sensitivity of real time PCR, it has become the method of choice for expression analysis study in various cells.

For the present study, expression of erythropoietin was quantitatively analysed at transcriptional level using real time PCR technique. Expression of EPO was normalized with the expression of endogenous control gene (ribosomal protein L25) as the expression of RP L25 gene was found constant and stable in different tissues or leaves of *N. tabacum* (Schmidt and Delaney 2010). Specific primers were designed for cDNA sequence of the EPO and RP

L25 transgenes using primer express software V 3.0. The primer sequences for cEPO and RP L25 have been mentioned in **Appendix VI**.

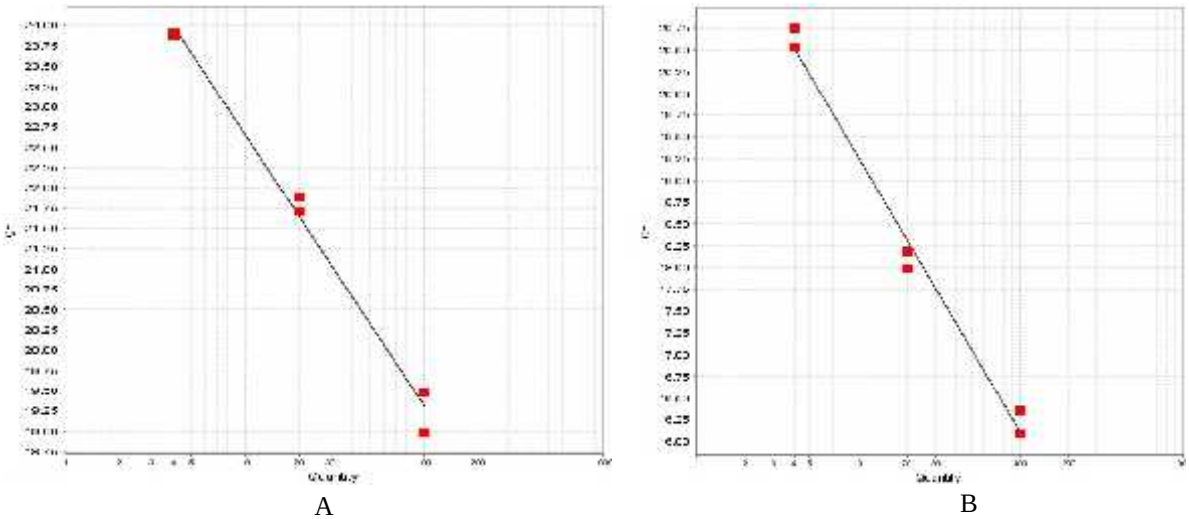
(a) PCR efficiency analysis

PCR amplification efficiency was estimated by the slope of standard curve. A standard curve slope of –3.32 indicates a PCR reaction with 100% efficiency. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of CT value vs. log of input cDNA concentration. Slope more negative than –3.32 indicates reaction is less efficient than 100%, efficiency and slope lesser negative than -3.32 represents reaction is more efficient than 100 %.

Formula for PCR efficiency calculation

$E=10^{(-1/slope)}-1$

PCR efficiency calculated for endogenous control gene L25 and target EPO using specific primers (mentioned in **Appendix VI**). Three different dilutions were taken and each dilution was used in the duplicates. PCR reaction mixture was prepared and PCR conditions were set as mentioned in **Section 3.2.4.9c** and **3.2.4.9d** respectively. The obtained PCR efficiency was 99.461% (slope -3.335) for endogenous control RP L25 and 107.303% (slope -3.158) for target EPO **(Figure 4.45)** respectively.



A B

Figure 4.45: A: Standard curve for PCR amplification efficiency of RP L25 Target: RP L25, % Efficiency: 99.461%.
B: Standard curve for PCR amplification efficiency of EPO Target: EPO, % Efficiency: 107.303%

PCR efficiency of the target and endogenous control under the acceptable range has proven the specificity of the primers towards its target sequence.

b) mRNA expression analysis

PCR reaction mixture of 10 µl/well was prepared containing 1X sybr green (5µl), 300 nM of transgene specific forward (0.3µl) and reverse primers (0.3µl). Final volume of reaction mixture was made up with sterile water **(Section 3.2.4.7c).** cDNA samples of transformed and non-transformed plants prepared in previous experiment were subjected to 1:5 dilution with sterile water. 5µl of diluted cDNA of each sample (in duplicates) were transferred to 48 well plate to 10µl of previously prepared reaction mixture was added as per the experimental design. Plate was properly sealed with the cap and kept in the instrument. Experiment was setup in the software connected to the instrument and method was run **(Section 3.2.4.9d).** Ct values obtained for the test samples (transformed with test expression vector pFGC5941Syn7cEPO) were compared with the Ct values obtained for the control plant sample (transformed with control expression vector pFGC5941cEPO) which indicates difference in the expression level of cEPO (**Figure 4.46a and b).** Expression of erythropoietin was normalized with expression of internal control gene ribosomal protein L25 (RP L25).

Formula for ΔΔCt calculation

Step 1

Normalization to endogenous control

Ct target gene- Ct endogenous gene=**Δ**Ct

Step 2

Normalization to calibrator sample

ΔCt sample- **Δ**Ct calibrator=**ΔΔ**Ct

Step 3

Finally ΔΔCt value put in the following equation.

$2^{-\Delta\Delta Ct}$

In previously reported study, it was found that RP L25 gene is one of the most stable reference genes for the real time PCR analysis as its expression does not get influenced by any stress (Schmidt and Delaney 2010).

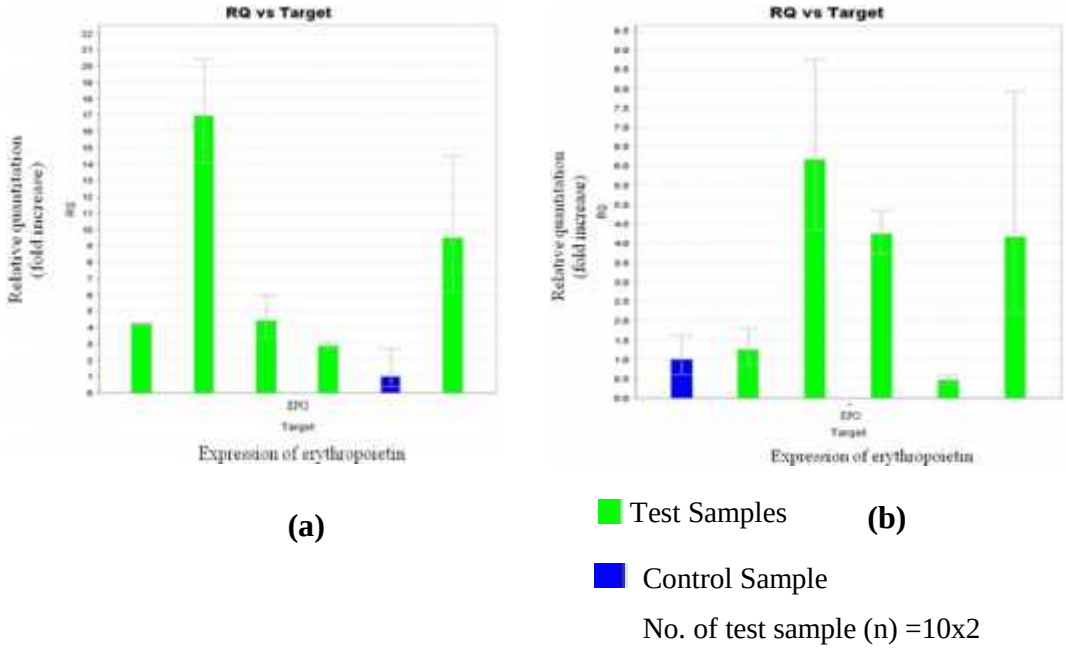


Figure 4.46 (a) and (b): mRNA expression analysis of EPO in test sample transformed with pFGC5941Syn7cEPO as compare to control sample transformed with pFGC5941cEPO; Error bars indicate standard deviation; RQ indicates Relative Quantitation.

Analysis of expression of cEPO in all ten test samples (plants transformed with pFGC5941Syn7cEPO) was compared with the expression of cEPO in control samples (plants transformed with pFGC5941cEPO). Green bars represent test samples and blue bars represent control samples **(Figure 4.46a and b).** Each sample was taken in duplicate and analysed.

As compared to the control samples around 2 to 17- fold increases was observed in the expression of erythropoietin at transcriptional level which revealed that expression of erythropoietin was enhanced in the presence of Syn7 intron.

EPO mRNA expression was qualitatively and quantitatively found higher in the presence of intron Syn7. These results indicated that presence of intron significantly increases the expression of erythropoietin at transcriptional level.

4.3.4 Analysis of protein expression using ELISA assay

Erythropoietin was quantified in plant extract of non-transformed and transformed plants of *N. tabacum* with pFGC5941SyncEPO (test vector) and pFGC5941cEPO (control vector) using ELISA Assay.

Total plant protein was extracted by protocol as mentioned in **Section 3.2.4.10**a. Total extracted protein was estimated using Bradford assay as mentioned in **Section 3.2.4.10b.** Presence of erythropoietin protein in the extract was confirmed by the ELISA Assay using EPO specific antibodies. Polyclonal IgG antibody as a primary and Goat-anti-rabit IgG as a secondary antibody.

Randomly selected, 3 transgenic plants transformed with control expression vector and 3 with test expression vector were selected for total protein extraction. 3 non-transformed plants of *N. tabacum* were also taken as control samples for total protein extraction. Sample of each plant extract was transferred in triplicates to ELISA plate (96 wells). ELISA assay was performed as mentioned in **Section 3.2.4.10c.** The qualitative results of the assay were calculated quantitatively using calibration curve of EPO standard protein. The linear calibration curve of EPO protein was generated using five dilutions of the erythropoietin standard ranging from 1.68 ng (0.1 IU) to 20.16 ng (1.2 IU) **(Figure 4.47a, Table 4.7)**. Readings obtained (at 450 nm) for the samples were subtracted with the reading of blank samples for normalization **(Table 4.6)**. Calibration curve was plotted with concentration of EPO against absorbance. The concentration of EPO in unknown samples was calculated from the known concentration of EPO standard solution plotted on standard curve (**Table 4.5**). Linearity was obtained in standard curve against the different concentration of EPO standard with regression coefficient 0.999.

Table 4.4: ELISA reading of EPO in known standard samples

S. No.	EPO (IU)	EPO (ng)	Readings for standard curve			Mean	Normalized Mean
1	0.0	0.000	0.120	0.129	0.134	0.128	0.000
2	0.1	1.680	0.378	0.302	0.287	0.322	0.195
3	0.3	5.040	0.599	0.480	0.368	0.482	0.355
4	0.6	10.08	0.665	0.820	0.737	0.741	0.613
5	0.9	15.12	0.956	0.977	1.010	0.981	0.853
6	1.2	20.16	1.126	1.067	1.400	1.198	1.070

Table 4.5: ELISA reading of EPO in unknown samples

S. No.	*Sample		Reading		Mean	Normalized Mean
1	N1	0.152	0.178	0.172	0.167	0.039
2	N2	0.170	0.260	0.141	0.190	0.062
3	N3	0.231	0.122	0.182	0.178	0.050
4	pF1	0.346	0.349	0.366	0.354	0.226
5	pF2	0.346	0.364	0.374	0.361	0.233
6	pF3	0.316	0.306	0.358	0.327	0.199
7	pSE1	1.173	1.122	1.158	1.151	1.023
8	pSE2	1.077	1.047	1.062	1.062	0.934
9	pSE3	1.071	1.054	1.126	1.084	0.956

*N= Non-transformed plants; pF= transformed plants with pFGC5941cEPO; pSE Transformed plants with pFGC5941Syn7cEPO.

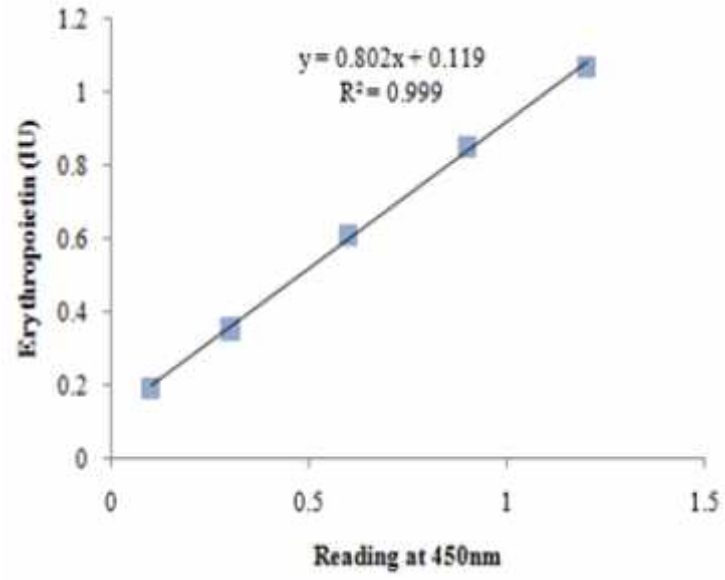


Table 4.6: Concentration of erythropoietin used for the standard curve preparation

S. No.	EPO in IU	EPO in ng
1	0.1	1.68
2	0.3	5.04
3	0.6	10.08
4	0.9	15.12
5	1.2	20.16

Figure 4.47a: Calibration curve for EPO concentration ranging from1.68 ng -20.16 ng

In the present study, concentration of EPO was calculated 2.124 ng/5µg of the total protein concentration for the plant transformed with pFGC5941cEPO (control expression vector).
In case of transformed plants with pFGC5941Syn7cEPO (test expression vector), concentration of erythropoietin was obtained 18.058 ng/5µg of total protein concentration **(Figure 4.47b).**

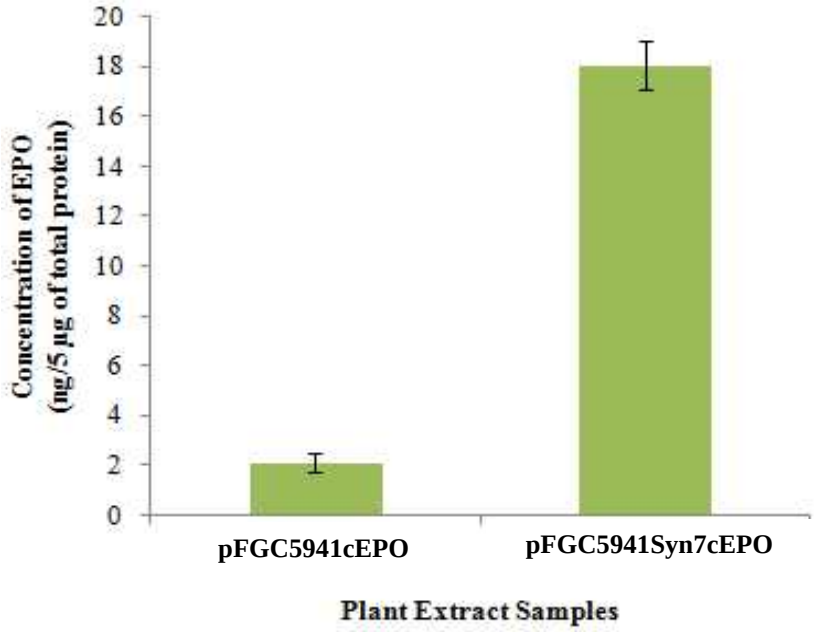


Figure 4.47b: ELISA assay for the comparative quantitative analysis of EPO in control expression vector treated plant extract and test expression vector treated plant extract. Student t-test was applied for the comparative analysis of EPO between two groups) Error bar indicates standard deviation; (***) or ($p<0.0001$) indicates the level of significance.

Comparative analysis was done by applying t-test between samples of two group treated with pFGC5941cEPO and pFGC5941Syn7cEPO constructs. Significantly increase with ($P<0.0001$) was found in EPO concentration in test samples.

Expression of erythropoietin at translational level or protein level was found higher in the plants transformed with test expression vector (pFGC5941Syn7cEPO) as compared to control vector (pFGC5941cEPO). Significantly higher, erythropoietin levels as indicated by ELISA results confirmed that intron Syn7 efficiently upregulate the expression of erythropoietin in transgenic plants which was observed in term of around ~8.5 fold increase in the expression

of erythropoietin at protein level in plants transformed with test vector having intron construct. The results of present study can be supported by the findings of Matsumoto el al reported in 1993 and 1995, the expression of EPO with strong CaMV35S promoter in BY2 cell lines of tobacco to be 1pg/g and 13.1 µg/6.3 kg of cells respectively (Matsumoto et al. 1993; Matsumoto et al. 1995). The present study reports enhanced expression of EPO in *N. tabacum* using Syn7 intron with yield of 7.223µg/g of fresh leaves. A similar study in moss reported increased expression of EPO (250 µg/g of dry weight of moss) using intron (Weise et al. 2007). The high expression in the moss as compared to present study in tobacco can be attributed to the use of protoplast culture with transient expression in contrast to transgenic plants of tobacco. A higher level of expression of EPO was seen at transcriptional level (2-17 fold increase) in our study also but it could not translate to higher expression at translational level. This need to be further investigated.

Recently, Jez et al. (2013) observed enhanced expression of rhEPO in tobacco (85 mg rhEPO/kg of fresh leaf) with the viral expression vector, which is established to the higher expression of transgene.

The present study evinced that intron has potential to upregulate the expression of transgene up to significant level.

Chapter 5

Summary and Conclusion

The project was designed to enhance the expression of erythropoietin protein in *Nicotiana tabacum* plant using intron-mediated enhancement strategy. IME strategy involves the insertion of proper intron at suitable site with transgene to regulate the transgene's expression.

In the present project, regulatory role of a synthetic intron **'Syn7'** was explored for the up regulation of expression of **erythropoietin protein** (a protein of human origin) in ***Nicotiana tobacum*** plant (a plant host system). The intron Syn7 was inserted between the promoter (CaMV35S) and coding region of cEPO as this site of insertion was reported to be very efficient in up regulation of transgene expression mediated by intron.

Two expression vectors namely **test expression vector** (pFGC5941Syn7cEPO) having intron Syn7 present between the promoter and cEPO, and **control expression vector** (pFGC5941cEPO) consists of only cEPO sequence were constructed to prove the hypothesis of the project. Restriction endonuclease digestion and ligation techniques were used to construct both the expression vectors. Confirmation of constructed vectors was done by PCR amplification technique using transgene specific primers, and also with restriction endonuclease digestion technique. Final confirmation of both the constructed vectors was performed by the sanger dideoxy sequencing technique.

Further, both the vectors were used to transform *Agrobacterium tumefaciens* LBA4404 cells which were in turn used to transform *N. tabacum* plant. Transformed plants were selected with the help of Bar gene expression as plant selection marker gene in transformed plants on MS medium containing 1 µg/ml concentration of basta and 500 µg/ml of cefotaxine. To ensure the absence of *Agrobacterim* in transformed plants cefotoxine antibiotic was used throughout the experiment. Transformed plants were maintained in both the *in vitro* and *in vivo* conditions during experimental period.

At molecular level, integration of transgene in genomic DNA was analyzed and confirmed by PCR amplification technique and southern blot technique. Metabolic burden was also

observed by the analysis of nicotine content in transformed *N. tabacum* plants using HPLC technique. Nicotine content was found to be higher in the transformed plants as compared to the non-transformed plants. This kind of fluctuation in the secondary metabolite content was also an indication of transformed plants.

The higher level (~2-17 fold) of mRNA transcript of erythropoietin gene with intron as compared to that of without intron as evident by real time PCR experiment indicate that presence of intron does influence the expression of transgene specifically when it is inserted as right place. Enhanced expression (~ 8.5 fold) level of erythropoietin was further confirmed at protein level by ELISA technique.

The results obtained in the present study revealed the significant increase in the expression of erythropoietin at mRNA and protein level in the presence of Syn7 intron as compared to the intron-less construct. The study indicates that presence of intron drastically influence the expression of transgene if designed and placed at appropriate location with or within transgene by its regulation. Hence, results of the study also prove our hypothesis of 'Intron (Syn7) mediated enhancement of expression of heterologous protein (erythropoietin) in plant (*N. tabacum*).

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*Cross references

Appendix I

(A) Extraction buffers for plasmid preparation

Alkaline Lysis solution- 1

1 M glucose 5 ml

1 M Tris-Cl (pH 8) 2.5 ml

0.5 M EDTA (pH 8) 2.0 ml

Final volume was made 100 ml with M.Q water and solution was autoclaved for 15 min at 15 psi on liquid cycle, and store at 4ºC.

Alkaline Lysis solution - 2

10 N NaOH 2 ml

10 % SDS 10 ml

Final volume was made 100 ml with M.Q water and use at room temperature.

Alkaline Lysis solution - 3

5 M potassium acetate 60.0 ml

Glacial acetic acid 11.5 ml

M.Q water 28.5 ml

Final volume was made 100 ml with M.Q water and solution was stored at 4ºC.

(B) Preparation of Equilibrated phenol

Phenol crystals were melted and the molten phenol was distillated. Prior to distillation 8-hydroxyquinoline was added at a final concentration of 0.1% (w/v). After distillation the distilled phenol can be stored at - 20^{o}C. As needed the phenol was removed and equal volume of 0.5 M Tris-Cl buffer pH 8 was added and stirred on the magnetic stirrer for 15 minutes. Then the aqueous and the solvent layers were allowed to separate. When the two phases were separated, the aqueous (upper) layer was aspirated using the glass pipette attached to a vacuum line equipped with appropriate traps. Then equal volume of 0.1 M Tris-Cl buffer pH 8 was added and again stirred on the magnetic stirrer for 15 minutes. Once again the two layers were allowed to separate and the aqueous (upper) layer was aspirated. The pH of the phenol layer was measured using the pH strips. The process was repeated till the pH of the

phenolic phase was shifted to > 7.8. After the desired pH, the final aqueous phase was removed and 0.1 M Tris-Cl buffer (pH 8) containing 0.2% mercaptoethanol was added to the equilibrated phenol. Equilibrated phenol was stored under the layer of 0.1M Tris-Cl pH 8 in amber coloured glass bottle at 4^{o}C for further use (Sambrook et al. 2007).

Appendix II

SOB Medium

Tryptone	20 gm
Yeast extract	5 gm
NaCl	0.5 gm

Solutes were completely dissolved by adding small amount of water and then 10 ml of 250 mM KCl was added. pH of the medium was adjusted to 7 with 5N NaOH. And volume of the solution was mad 1 litre with M.Q water. Solution was autoclave for 20 minutes at 15 psi on liquid cycle. Just before use, 5 ml of a sterile solution of 2 M MgCl2 was added.

SOC medium

Identical to SOB medium except it contains 20 mM glucose.

Appendix III

50x TAE (For Agarose gel)

24.2 gm of Tris base

5.71 ml of glacial acetic acid

10 ml of 0.5 M EDTA (pH 8)

Final volume was made 100 ml with M.Q water Solution was stored at 4ºC

6x gel loading dye

0.25 % (w/v) bromophenol blue

30 % (w/v) glycerol

10X Tris EDTA Buffer, pH8.0

100mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0)

Sterile solution by autoclaving for 20 mins at 15psi. store the buffer and room temp.

Tris-Cl, pH 8.0

Dissolve 12.1g of Tris-base in 800ml of H2O. Adjust pH to the desired value by adding concentrated HCl around 42 ml.

Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 litre with H2O. dispense in to aliquots and sterilize by autoclaving.

EDTA (0.5M, pH 8.0)

Add 186.1g of disodium EDTA.2H2O to 800 ml of H2O. stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (20 g of NaOH pellets). Dispense in to aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go in to solution until the pH of the solution is adjusted to 8.0 by the addition of NaOH.

Ethidium bromide (10mg/ml)

Add 1 g of ethidium bromide to 100 ml of H2O. stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Store the solution at dark conditions.

Appendix IV

10x FA gel buffer

200 mM 3-[N-morpholino] propanesulfonic acid (MOPS)

50 mM sodium acetate

10 mM EDTA

pH to 7.0 with NaOH

1x FA gel running buffer

100 ml 10x FA gel buffer

20 ml 37% (12.3 M) formaldehyde

880 ml RNase-free water

5x RNA loading buffer

16 µl saturated aqueous bromophenol blue solution

80 µl 500 mM EDTA, pH 8.0

720 µl 37% (12.3 M) formaldehyde

2 ml 100% glycerol

3.084 ml formamide

4 ml 10 x FA gel buffer

RNase-free water to 10 ml

RNA bands were visualized under UV illumination

Appendix V

Southern blot

20X SSPE Buffer

NaCl	175.3 g
$NaH_2PO_4.H_2O$	27.6 g
EDTA	7.4 g

All the components were dissolved in 800 ml milli Q water. 6.5 ml of 10N NaOH was added to adjust the pH 7.4. Final volume was made up to 1000 ml with milli Q water.

Buffer preparation for colour development of membrane

Buffer 1

Maleic Acid Buffer (Buffer 1-Roche), 100mM Maleic Acid, 150 mM NaCl, H2O was added to adjust desired volume.

pH was adjusted up to 7.5 using NaOH solution.

Buffer 2

Buffer 2 was prepared by dissolving 1% (w/v) blocking reagent (Roche) with buffer approximately 1 hour before use. Blocking reagent was dissolved in buffer 1 at 50 to 70^{o}C and stirred vigorously.

Buffer 3

100 mM Tris-base, 100 mM NaCI, 50 mM MgCI2.6H20 and H_2O was added to adjust final volume, pH 9.5 was adjusted with HCl

Colour Detection: Ready to use 200 µl of NBT solution was added in 10 ml Buffer 3.

Appendix VI

Primers for PCR

S. No.	Gene	Primer sequence	Amplicon (bp)
1	RP L25	FP 5′ TCGTATTAGTGCACCTGGAA3′	160
		RP 5′CTTCACGGCATCCTTAATCT3′	
2	BAR	FP 5′GAAGTCCAGCTGCCAGAAAC3′	246
		RP 5′AGTCGACCGTGTACGTCTCC3′	
3	Syn7	FP 5′GAGAGGACACGCTCGAGTATAAG3′	394
		RP 5′ACAGCCAGGCAGGACATTC3′	
4	EPO	FP5′CCACCACGCCTCATCTGTGAC3′	508
		RP5′TCTGTCCCCTGTCCTGCAGGC3′	

Primers for Real time PCR

S. No.	Gene	Primer sequence	Amplicon (bp)
1	EPO	FP5′TTCGCAGCCTCACCACTCT3′	112
		RP5′CGGAAAGTGTCAGCAGTGATTG3′	
2	L25	FP5′AGAAGATTAAGGATGCCGTGAAGA3′	123
		RP5′CCAAAGCATCATAGTCAGGAGTCA3′	

Primers for amplification of constructed part of vector (for sequencing)

S. No.	Gene	Primer sequence	Amplicon (bp)
1	Vector	FP5′TAAGGGATGACGCACAAT3′	1147
		RP5′TTTTACAACGTGCACAACAG3′	

Appendix VII

Aligned sequences of constructed vectors using Multiple Alignment software CLUSTALW

Publications and Presentations

Research Articles

- Sonal Sharma, Poojadevi Sharma, **Sheetal Yadav**, Indira Purohit, Anshu Srivastava, Astha Varma, Neeta Shrivastava (2015) Homogenous PCR of heterogenous DNA from Phenolic rich barks of Terminalia species for DNA based adultration detection. *Proc Natl Acad Sci, India Sect B: Biol Sci*, DOI:10.1007/s40011-015-0576-z.
- **Sheetal Yadav**, Poojadevi Sharma, Anshu Srivastava, Priti Desai, Neeta Shrivastava (2014) Strain specific Agrobacterium-mediated genetic transformation of Bacopa monnieri. *Journal of Genetic Engineering and Biotechnology*, 12 (2):89-94.
- Poojadevi Sharma, **Sheetal Yadav**, Anshu Srivastava, Neeta Shrivastava (2013) Methyl jasmonate mediates upregulation of bacoside A production in shoot cultures of Bacopa monnieri. *Biotechnol Letters*, 35(7):1121-5.

Book Chapter

- Poojadevi Sharma, Sonal Sharma, **Sheetal Yadav**, Anshu Srivastava, Indira Purohit, and Neeta Shrivastava. "Plant Derived Bioactive Molecules: Culture Vessels to Bioreactors." *In Production of Biomass and Bioactive Compounds Using Bioreactor Technology*, pp. 47-60. Springer Netherlands, 2014

NCBI submissions

- Sonal Sharma, Poojadevi Sharma, Sheetal Yadav, Anshu Srivastava, Neeta Srivastava. Accession no **KF925433**: Saraca asoca 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence.

Poster presentations

- Sonal Sharma, Poojadevi Sharma, **Sheetal Yadav,** Indira Purohit, Neeta Shrivastava.PCR inhibitors and enhancers in structural genomic studies in Medicinal plants. National Conference on Herbal Drug Research: Challenges and Opportunities, November 5-7, 2014, Ahmedabad, India. **(3rd Price).**
- Poojadevi Sharma, Sonal Sharma, **Sheetal Yadav,** Anshu Srivastava, Neeta Shrivastava Integrative quality control approach for Indian herbal raw drug ‘Asoka chhal’. 18th Annual Convention of Society of Pharmacognosy on *Promotion and*

Globalisation of Indian Herbal Products- Perspectives and Prospects, February 21-22, 2014.Rohtak, Haryana, India .

- Sonal Sharma, Poojadevi Sharma, **Sheetal Yadav,** Anshu Srivastava, Astha Varma, Neeta Shrivastava. Structural genomics as a species specific quality control tool for highly traded bark of *Terminalia arjuna*. 18th Annual Convention of Society of Pharmacognosy on *Promotion and Globalisation of Indian Herbal Products-Perspectives and Prospects*, February 21-22, 2014 Rohtak, Haryana, India.
- **Sheetal Yadav,** Poojadevi Sharma, Anshu Srivastava, Priti Desai and Neta Shrivastava. Agrobacterium strain-cultivar compatibility for genetic transformation of *Bacopa monnieri*. National Symposium on Impact of Plant Tissue Culture on Advances in Plant Biology and 33rd PTCA Meet, January 19-21, 2012, Ahmedabad, Gujarat, India.
- Poojadevi Sharma, **Sheetal Yadav,** Anshu Srivastava and Neeta Shrivastava. Methyl jasmonate mediated induction of Bacoside A production in *Bacopa monnieri* (L.) Pennell: An indication of its influence on the terpenoid biosynthetic pathway. International Conference on New Developments in Drug Discovery from Natural Products and Traditional Medicines (DDNPTM), November 20-24, 2010, Mohali, Chandigarh, India.