BOLU ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

ANALYSES OF PROMOTERS IN *ARABIDOPSIS THALIANA* OF *3 BETA HSD* AND *5 BETA POR* GENES ISOLATED FROM DIGITALIS SPECIES

MASTER OF SCIENCE

NOREEN ASLAM

BOLU, JUNE 2018

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APPROVAL OF THE THESIS

"ANALYSES OF PROMOTERS IN *ARABIDOPSIS THALIANA* OF 3 BETA HSD AND 5 BETA POR GENES ISOLATED FROM DIGITALIS SPECIES" submitted by **Noreen ASLAM** in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Biology, The Graduate School of Natural and Applied Sciences of ABANT IZZET BAYSAL UNIVERSITY in 16/05/2018** by

Examining Committee Members	**Signature**
Supervisor Prof. Dr. Ekrem Gürel ABANT IZZET BAYSAL UNIVERSITY	.....
Member Assoc. Prof. Dr. Mehmet ÖZTÜRK ABANT IZZET BAYSAL UNIVERSITY	.....
Member Assoc. Prof. Dr. Mehmet Cengiz BALOĞLU KASTAMONU UNIVERSITY	.....

Graduation Date : 26.06.2018

Doç. Dr. Ömer ÖZYURT .....

Director of Graduate School of Natural and Applied Sciences

This work is dedicated to my late mother, Zikran Aslam, and beloved father, Mian Muhammad Aslam, for their great love and support.

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Noreen ASLAM

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ABSTRACT

ANALYSES OF PROMOTERS IN *ARABIDOPSIS THALIANA* OF *3 BETA HSD* AND *5 BETA POR* GENES ISOLATED FROM DIGITALIS SPECIES

MSC THESIS
NOREEN ASLAM
ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. EKREM GÜREL)

BOLU, JUNE 2018

Digitalis ferruginea, known as foxglove, is famous for the production of secondary metabolites; i.e., cardiac glycosides. The progesterone *5β-reductase* (*5βPOR1 and 5βPOR2*) and *3β-hydroxysteroid dehydrogenase* (*3β-HSD*) are important key step genes in the pathway of biosynthesis of cardenolide in *Digitalis* species. For the first time, promoters of *3β-HSD, 5βPOR1* and *5β POR2* were isolated from genomic DNA of *Digitalis ferruginea* and sequence analysis was performed. Sequence information of the promoters revealed that these promoters have abundant *cis*-elements related to light responses. *5βPOR1promoter* specific *cis*-elements are circadian and heat shock. *5βPOR2promoter* specific regulatory elements are defense, stress and methyl jasmonate. Wound response element was found specifically in the *3β-HSDpromoter*. Functional characterization of the *3β-HSDpromoter*::GUS in Arabidopsis plants transformed by floral dip transformation showed activity in cotyledons, newly emerged leaves, vascular tissues of shoot and root, trichomes, shoot apical meristem (SAM) and hydathodes during plant growth and developmental stages. Under the treatments of 3% sucrose or 2% mannitol intensively enhanced GUS activity in the seedlings. In the presence of NaCl (150 mM) promoter expression activity was downregulated. *5βPOR1promoter*::GUS activity was the highest in 5 and 10 days old seedlings in all tissues except root apical meristem, then decreased in 15 days old seedlings. *5βPOR2promoter*::GUS activity was confined to vascular tissues in cotyledons in 5 and 15 days old seedlings. In 10 days old seedlings, promoter activity was intensively found in cotyledons, vascular tissues of cotyledons, hypocotyl and root adjacent to hypocotyl. Promoter activity was intensive in hypocotyl of 15 days old seedlings. The promoter sequences and functional characterization in Arabidopsis, revealed that the genes are light regulated and the *3β-HSD* have role in plant growth, development and abiotic stress tolerance. Further, the genes *5βPOR1* and *5βPOR2* also have crucial role in normal plant growth during developmental stages.

KEYWORDS: Promoters, Sequences, *5βPOR1, 5βPOR2 and 3β-HSD,* Functional characterization, Arabidopsis, Growth and development, GUS, Stress

ÖZET

DIGITALIS TÜRLERINDEN IZOLE EDILECEK 3 BETA HSD VE 5 BETA POR GENLERINE AIT PROMOTORLARIN ARABIDOPSIS THALIANA'DA ANALIZI
YÜKSEK LISANS TEZI
NOREEN ASLAM
ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
BIYOLOJI ANABILIM DALI
(TEZ DANIŞMANI: PROF. DR. EKREM GÜREL)

BOLU, HAZİRAN - 2018

Yüksükotu olarak bilinen *Digitalis ferruginea,* kardiyak glikozitlerin üretimi için kullanılmaktadır. Progesteron 5β-redüktaz (*5βPOR1* ve *5βPOR2*) ve 3β-hidroksisteroid dehidrojenaz (*3β-HSD*) digitalis türlerinde kardenolidin biyosentez yolundaki önemli anahtar basamak genleridir. Bu çalışmada *3β-HSD, 5βPOR1* ve *5βPOR2* promotörleri Digitalis ferruginea'nın genomik DNA'sından İlk kez izole edilip, dizi analizi yapılmıştır. Promoterlerin sekans bilgileri, bu promoterlerin ışık tepkileriyle ilgili bol cis-elementlere sahip olduğunu ortaya çıkarmıştır. *5βPOR1promoter* spesifik cis elemanları sirkadiyen ve ısı şokudur. *5βPOR2promoter* spesifik düzenleyici elemanlar savunma, stres ve metil jasmonattır. Yara yanıt elementi, *3β-HSD*promoter'da spesifik olarak bulunmuştur. Çiçek dalma transformasyonu ile transform olmuş Arabidopsis bitkilerinde *3β-HSDpromoter* :: GUS'un fonksiyonel karakterizasyonu, kotiledonlarda, yeni ortaya çıkan yapraklarda, filiz ve kök vasküler dokularında, trikomlarda, filizlenen apikal meristemde (SAM) ve bitki büyümesi ve gelişim aşamalarında kist hidatodlarda aktivite göstermiştir. Fidelerde% 3 sukroz veya% 2 mannitol bulunduran ortamda yoğun GUS aktivitesi izlenmiştir. NaCl (150 mM) promoter ekspresyon aktivitesinin mevcudiyetinde downregüle aktivite izlenmiştir. *5βPOR1promoter* :: GUS aktivitesi, kök apikal meristem hariç tüm dokularda 5 ve 10 günlük fidelerde en yüksek iken 15 günlük fidelerde azalmıştır. *5βPOR2promoter* :: GUS aktivitesi 5 ve 15 günlük fidelerde kotiledonlarda vasküler dokularla sınırlıydı. 10 günlük fidelerde, kotiledonlarda, kotiledonların vasküler dokularında, hipokotil ve hipokotile bitişik kökte promotör aktivitesi yoğun olarak izlenmiştir. 15 günlük fidelerin hipokotilinde yoğun promoter aktivitesi görülmüştür. Arabidopsis'te promotör sekansları ve fonksiyonel karakterizasyon, genlerin hafifçe düzenlendiğini ve *3β-HSD*'nin bitki büyümesinde, gelişiminde ve abiyotik stres toleransında rol oynadığını ortaya koymuştur. Ayrıca, *5βPOR1* ve *5βPOR2* genleri de gelişim aşamaları sırasında normal bitki büyümesinde önemli rola sahiptir.

ANAHTAR KELİMELER: Promotorlar, Sekanslar, *5βPOR1, 5βPOR2 and 3β-HSD*, Fonksiyonel karaketrizasyon, Arabidopsis, Büyüme ve gelişme, GUS, Stres

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LIST OF ABBREVIATIONS AND SYMBOLS

5βPOR1	**:** Progesterone *5β-reductase* 1
5β POR2	**:** Progesterone *5β-reductase* 2
3β-HSD	**:** *3β-hydroxysteroid dehydrogenase*
bp	**:** Base pair
gDNA	**:**Genomic DNA
GUS	**:** β-Glucuronidase
kbp	**:** Kilo base pair
OD	**:** Optical density
PCR	**:** Polymerase chain reaction
µM	**:** Micro molar
mM	**:** Milli molar
M	**:** Molar
TAE	**:** Tris-acetate-EDTA
TE	**:** Tris-EDTA
X-Gluc	**:** 5-Bromo-4-chloro-1H-indol-3-yl β-D-glucopyranosiduronic acid

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1. INTRODUCTION

Digitalis species (*ferruginea*, *lamarkii*, *purpurea*, *trojana*), known as foxglove, are famous for the production of secondary metabolites; cardiac glycosides. Cardiac glycosides are secondary metabolites which have pharmaceutical importance in cardiac arrest and also anti-cancer activities (Bertol et al. 2011; Kreis et al. 2014). The genes progesterone *5β-reductase* (*P5βR* or *5 beta POR*) and *3β-hydroxysteroid dehydrogenase* (*3β-HSD*) are important key step genes in the pathway of biosynthesis of cardenolide in digitalis species. During last two decades the extensive research has been conducted on the cardiac glycosides, pathways and substrates for the glycosides, recombinant protein production of *5βPOR1* (Herl et al. 2006) and *3β-HSD* (Ernst et al. 2010) using bacterial heterologous system of expression. The cardenolides biosynthesis also known to be triggered due to *5βPOR2* mediated by the stresses like heat, cold, wound, submergence in water, hydrogen peroxide (H_2O_2), precursor of ethylene biosynthesis: 1-aminocyclopropane-1-carboxylic acid (ACC), drought and nutrient deficiency in soil (PérezBermúdez et al. 2010).

The sequence information of these genes is available in National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). However, the promoter sequences of these genes are not available and so the functional characterization.

The promoter is the DNA sequence or fragment which is located upstream of the gene (Figure 1.1) that direct the transcription of the gene. It is the promoter fragment which directs the transcription of the gene to which extent when and where conditions. In promoter fragment, DNA elements or *cis*-acting elements are present for the legitimate initiation and regulation of transcription of the gene. These *cis*-elements are core promoter, long-range elements and proximal elements. The core promoter is the key DNA sequence which serves as recognition site for the RNA polymerase II and other elements to initiate the transcription. TATA box a highly conserved element present in all eukaryotic promoters (Joshi 1987), the initiator element and the downstream elements are some of the known core promoter elements (Allison, 2007). The enhancer element which is regulatory element activate

the gene expression. CAAT box is a consensus sequence close to -80 bp from the start point (ATG). It plays crucial role in the promoter efficiency by increasing its strength and it seems to function in either orientation. GC box is sequence rich in guanidine (G) and cytidine (C) nucleotides and usually found in multiple copies in the promoter surrounding the TATA box. CAP site is a transcription initiation point from where transcription usually starts (Allison, 2007).

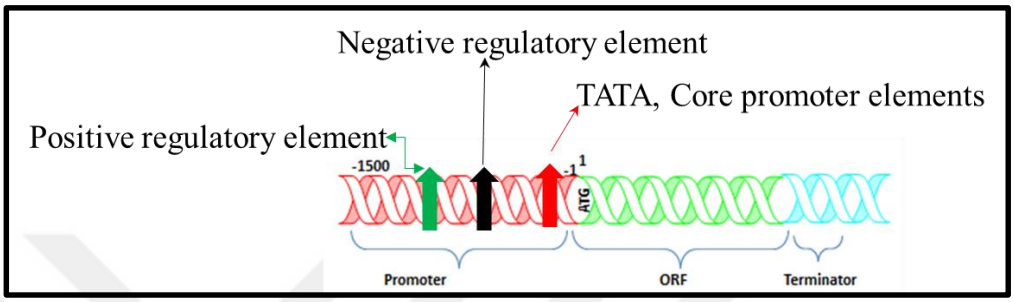

Figure 1.1. Typical structure of eukaryotic promoter along with gene and terminator fragments.

The composition and organization of these regulatory elements in the promoter defines the transcription and expression of the gene under specific environment. For example, enhancer element boosts up the transcription synergetic interactions with transcriptional activators (independently or in combinations), contributing in developmental, environmental or hormonal controls. Contrarily, the negative regulatory elements, by interaction with transcriptional repressors, inhibit gene expression. The multilateral interactions of trans acting factors, regulatory elements and basic transcription factors determine how, when and where to which extent a particular gene is regulated.

Promoter's *cis*- acting elements related to expression of the gene during normal plant growth and development and abiotic, biotic stress conditions may vary from gene to gene. In fact, these *cis*-elements defines the expression and role of the gene and making it unique and novel with respect to function from other gene family members.

Based on promoter's characteristic and their salient features, promoters can be divided into six different categories.

1.1 Constitutive promoters - promoters that function in all cells, tissues and organs through different stages of development, e.g. promoters of house-keeping genes, cauliflower mosaic virus (CaMV 35S) promoter, widely use in dicots for transgene expression studies. *APX*, *SCP1*, *PGD1*, *R1G1B*, and *EIF5* are the constitutive promoters which have been reported for transgene expression in rice (Park et al., 2010).

1.2 Tissue-specific promoters - promoters that direct expression in specific tissues only, e.g., pollen, phloem, xylem, embryo, tapetum and meristem; *PtrDP3*promoter is an example of phloem tissue specific promoter (Nguyen et al., 2017).

1.3 Cell-specific promoters - promoters that direct expression in specific cells, e.g., pollen and guard cells. Such as *APRS* promoter which pollen specific promoter (Jopcik et al., 2013).

1.4 Developmental stage-specific promoters - promoters that regulate expression of genes at specific developmental stages and particular organs, e.g., root, stem, leaf, flower, fruit and seed. *GhGDSLpro* is an example of cotton boll specific promoter (Yadav et al., 2017).

1.5 Hormone-regulated promoters - promoters that induce gene expression in response to phytohormones at different stages of plant growth, e.g., auxin-inducible promoter for which *CaMV 35S* is the best example (Sameeullah et al., 2013).

1.6 Environmental condition-controlled promotors - promoters that respond to external factors, e.g., heat, cold, light, UV, stress, salt and pathogen attack. *AtSUC3* promoter (Meyer et al. 2004) and *GRMZM2G315431* promoter (Li et al., 2017) are examples of wound and pathogen inducible promoters, respectively.

The SDR (Short-chain dehydrogenases/reductases) superfamily shows a very broad family of enzymes, most of which are recognized to be NAD-dependent or NADP-dependent oxidoreductases from *E. coli* to human. Since, the first characterized member of this family was the novel alcohol dehydrogenase in

Drosophila, so this family used to be termed as “insect-type” or “short-chain” alcohol dehydrogenase (Cheng et al. 2002).

Alcohol dehydrogenase (*ADH*) promoter is known to be expressed in root tissues and also during (deficiency of oxygen) hypoxia stress (Chung and Ferl 1999, Dolferus et al. 1994). Also, *ADH1promoter*::GUS activity shows its upregulated expression during abiotic stresses like drought, cold and ABA treatment (Dolferus et al. 1994, Hoeren et al. 1998, de Bruxelles et al. 1996). However, this promoter cannot be expressed during plant growth and developmental stages (Chung and Ferl 1999).

In Arabidopsis, 1.5 kb promoter of *AtHSD1* fused with the reporter gene GUS showed that the activity of promoter was highest in shoot parts of seedlings while weak expression was observed in root tissues under light and dark conditions. Additionally, the highest promoter activity was found in vascular tissues. Some researchers related these expression activities with the involvement of brassinosteroids in vascular differentiation (Sasse 2003). Further, they speculated that expression in vascular tissues may be related to stem thickness according to their previous observations. Moreover, the promoter activity was also observed in the bud and silique pedicels (Li et al. 2007).

AtSDR1promoter::GUS activity was studied in Arabidopsis under salt and cold treatment but no noticeable differences were observed except weak expression in vascular tissues. On the other hand, presence of 2% glucose or mannitol enhanced the GUS expression activity significantly, which was limited to the vascular bundles of cotyledons as well as to hypocotyls and roots. Further, 6% glucose arrested the plant development in the presence of ABA with enhanced GUS expression activity in cotyledons, hypocotyl and root tip. Nonetheless, 6% mannitol application to induce osmotic stress exerted broader and intense GUS expression activity in whole seedling with root (Cheng et al. 2002).

AtSDR3pro::GUS showed extreme expression activity in the radicle tip and the area around the cotyledon hydathode of the emerging seedlings of 2 days old. In eight days old seedlings, promoter activity was also observed in the lateral root primordia and lateral root tips. As compared to *AtSDR3pro*, *AtSDR1pro* activity was found in the vascular tissues of the cotyledon and roots of young (2 and 8 days old) seedlings (Hwang et al. 2012). *AtHSD1pro*::GUS expression activity was equally observed in the endosperm and embryo of mature seed. In embryo developmental

stages, GUS activity was found pale in early bent embryo, darker in maturing and later maturing stages. In germinating plantlets, GUS activity was detected pacifically in embryonic tissue but not in newly developing leaves (Baud et al. 2009). *OsHSD1pro*::GUS expression activity was found in coleoptiles and root elongation zones, mature leaf sheaths, stem, panicles and stames. However, promoter activity was absent in root meristems and seeds. The transverse section of young roots, stems, leaves and sheaths revealed strong expression activity in vascular tissues (Zhang et al. 2016).

1.7 *Arabidopsis thaliana* as a model plant for promoter functional analysis

Arabidopsis thaliana is a model plant with a short generation time of 8 weeks from seed to seed cycle. Due to its short stature (approximately 20 cm tall), it is easy to manage and grow at high density under growth room or glass house conditions. Due to its simple nature of genome structure (diploid), it is easy to use for the functional studies of the target promoters or genes. It is self-fertilized and thus easy to obtain homogenous transgenic seedlings within 2-3 generations. A single plant produces hundreds to thousands of seeds. It is also easy to transform it with the gene of interest for the functional studies by floral dip transformation and stable transformants can be obtained within about 6 weeks.

2. AIM AND SCOPE OF THE STUDY

The genes progesterone *5β-reductase* (*P5βR*) and *3β-hydroxysteroid dehydrogenase* (*3β-HSD*) are important key step genes in the pathway of biosynthesis of cardenolide in digitalis species. However, the expression patterns of these genes based on functional analyses of the promoters are not available in the literature for any of the digitalis plant species. Also, the promoter sequence of these genes are not available in the online public database of biotechnology information centers.

Therefore, the aim of this study was to clone the promoter fragments of the genes progesterone *5β-reductase* (*5βPOR1* and *5β POR2*) and *3β-hydroxysteroid dehydrogenase* (*3β-HSD*) from *Digitalis ferruginea*. In order to determine novel roles and functions of the genes, promoter sequences were then analyzed.

Further, transgenic Arabidopsis plants by stable transformation using floral dip transformation method were produced. The transgenic Arabidopsis plants carrying promoter fragments of *3β-HSDpro*::GUS, *5βPOR1pro*::GUS and *5βPOR2pro*::GUS were subjected to GUS histochemical staining during plant growth and development stages to determine promoters' activities in different tissues. In addition, the expression of 3*β-HSDpro*::GUS under abiotic stress assays were performed to determine the role of the 3*β-HSD* under the abiotic stress conditions. These promoters isolated from *Digitalis ferruginea* and their functional characterization studies were performed in *Arabidopsis thaliana* for the first time.

3. MATERIALS AND METHODS

3.1 Genomic DNA isolation

High quality gDNA (genomic DNA) was isolated by the method of Doyle (1990) from fully expanded leaves of *Digitalis ferruginea* (Figure 3.1). Promoter fragments of *3β-HSD*, *P5βR1* and *P5βR2* genes were cloned following the procedure as described by manufacturer GenomeWalker™ Universal Kit (Clontech, CA, USA).

3.2 Digestion of gDNA

In order to produce four gDNA libraries (DL), gDNA (0.1 µg/µl) was digested with four blunt end *Dra* I (DL1), *EcoR* V (DL2), *Pvu* II (DL3) and *Stu* I (DL4) restriction enzymes. Reaction mixture was prepared as shown in Table 3.1. Four tubes were prepared for the digestion of gDNA with each enzyme in 1.5 µL micro centrifuge tubes. Reaction mixture was mixed gently by inverting the tubes and incubated at 37°C for 2 h. Then, the reaction was vortexed at slow speed for 5–10 sec and then returned to 37°C overnight (16-18 h).

3.3 Agarose gel electrophoresis for DNA samples

Standard Agarose gels 1% were prepared using 1X TAE Buffer to check the high quality of DNA isolations and also restriction fragments and to separate the PCR products. 2 µl of 6X loading dye per 10 µl samples were loaded and the DNA was separated at 100 V.

3.4 Extraction of Gel for DNA samples

Extraction of gel and purification procedures for PCR products or restriction fragments were performed according to the Nucleospin protocol of the Gel Extraction Kit after electrophoresis of the samples in a 1% Agarose gel. DNA was

eluted with 30 µL elution buffer available in the kit. Ligation procedure was followed according to the instructions of TA cloning kit manual (Thermo Scientific, USA).

3.5 *E. coli* transformation

All ligation products (5µl, 100 ng) and Plasmids, pCR2.1 and pCambia 1381 (1 µl, 25 ng/µL) are transformed into competent Top10® *E. coli* by heat shock following the manufacturer's protocol (Thermo Scientific, USA). The cells were thawed on ice and after addition of the plasmids or ligation products they were incubated for 30 minutes on ice then followed by a heat shock treatment at 42°C for 30 seconds and five minutes incubation on ice. SOC medium (250 µl) was added to 50 µl of cell culture and incubated at 37°C for 1 hour with shaking at 175 RPM. 80 µl of the culture were plated on LB plates containing the appropriate antibiotics for selection and grown at 37°C overnight. PCR colonies were performed on single colonies and grown overnight in liquid LB-medium containing antibiotics, harvested and used for plasmid isolation and glycerol-stock.

Table 3.1. Reaction mixture for restriction digestion of genomic DNA

Restriction digestion reaction components	**Quantity**
Restriction enzyme each (*Dra* I (DL1), *EcoR* V (DL2), *Pvu* II (DL3) and *Stu* I (DL4) all are10 units/µL in separate tube	8 µL
Genomic DNA (0.1µg/µL)	25 µL
Restriction enzyme buffer	10 µL
Nuclease-free H_2O	57 µL
Total volume	**100 µL**

Figure 3.1. *Digitalis ferruginea* plant used for isolation of genomic DNA (AIBU campus, snap shot by Noreen Aslam)

3.6 Purification of digested gDNA

To each reaction tube, an equal volume (95 µL) of phenol was added. The sample was vortexed at slow speed for 5–10 sec. Then, the samples were spun down briefly at room temperature to separate the aqueous and organic phases. Upper (aqueous) layer was transferred into a fresh 1.5 mL tube. To each tube, an equal volume (95 µL) of chloroform was added. The samples were vortexed at slow speed for 5–10 sec. Then, the samples were spun down briefly at room temperature to separate the aqueous and organic phases. Using a pipette, the upper (aqueous) layer was transferred into a fresh 1.5-mL tube. The lower (organic) layer was discarded properly into the hazardous waste. To each tube, 2 volumes (190 µL) of ice cold 95% ethanol, 1/10 volume (9.5 µL) of 3 M NaOAc (pH 4.5), and 20 µg of glycogen were added.

Then, the samples were vortexed at slow speed for 5–10 sec. The samples were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was removed and pellet washed in 100 µL of ice cold 80% ethanol and centrifuged at 14,000 rpm for 10 min. Again, the supernatant was removed and the pellet air dried. Pellet was then dissolved in 20 µL of TE (10/0.1, pH 7.5). The samples were vortexed at slow speed for 5–10 sec. From each reaction tube, 1 µL DNA sample was removed and run on a 0.6% agarose/ EtBr gel to determine the approximate quantity of DNA after purification.

3.7 Ligation of digested genomic DNA to GenomeWalker™ Adaptors

Four gDNA libraries were ligated with GenomeWalker™ Adaptors (Clontech, CA, USA). From each tube, 4 µL of digested, purified DNA (700 ng/µL) was transferred to a fresh 0.5 mL tube. To each tube, ligation reaction mixture as shown in Table 3.2 was added and then incubated at 16 ºC overnight. To stop the reactions, the tubes were incubated at 70°C for 5 min and 72 µL of TE (10/1, pH 7.5) was added to each tube, and vortexed at slow speed for 10–15 sec.

3.8 PCR-based DNA Walking in GenomeWalker Libraries to clone promoter fragment of *3β-HSD* genes

3.8.1 Primary PCR

There are two PCR steps in isolation of promoters based on GenomeWalker libraries, i.e. the primary PCR then followed by secondary PCR. Gene specific primers were designed according to the instructions of the kit. For the promoter cloning of *3 β-HSD, P5βR1* and *P5βR2* two gene specific primers (GSP1 and GSP2) were designed for primary and secondary PCR reactions, respectively. GSP1 of *3 β-HSD, P5βR1* and *P5βR2* genes was used in separate reactions along with AP1 primer in primary PCR reaction. GSP1 primers are shown in Table 3.3.

Table 3.2. Ligation of Digested Genomic DNA to GenomeWalker™ Adaptors

Restriction digestion reaction components	**Quantity**
GenomeWalker Adaptor (25 µM)	1.9 µL
Digested, purified DNA (700 ng/µL)	4 µL
10X ligation buffer	1.6 µL
T4 DNA ligase (6 units/µL)	0.5 µL
Nuclease-free H_2O	2 µL
Total volume	**10 µL**

Table 3.3. Primers for cloning of promoter's fragment of the genes *3β-HSD, 5βPOR1* and *5βPOR2* using gDNA libraries

Primers	**Sequences (5' to 3') Reverse compliments**	**Tm**
3β-HSD-GSP1	5'-TCATCTCTGACGTCGCAGTGGTAGTAAC-3'	67°C
3β-HSD-GSP2	5'-CTGACGTCGCAGTGGTAGTAACTTATCTTG-3'	67°C
P5βR1-GSP1	5'-GACGTAATTGATCGGATTATCCTCATGC-3'	67°C
P5βR1-GSP2	5'-ATTGATCGGATTATCCTCATGCCAGG-3'	67°C
P5βR2-GSP1	5'-GGACTTGGATAGAGTTTCAGCCAGACC-3'	67°C
P5βR2-GSP2	5'-GATAGAGTTTCAGCCAGACCGCTTCC-3'	67°C

Gene specific primers (GSP) were designed from anti sense sequence of the gene to design reverse primer while forward primer is based on adaptor sequence and that forward primer was supplied by the kit.

GSP1 primer sequence of 3*β-HSD* was designed from full length ORF of *3β-HSD* (gene bank accession KM406483) from 206 nucleotide number to 179 nucleotide number as reverse compliment. GSP1 primer sequence of *P5βR1* designed from full length ORF of *P5βR1* (gene bank accession KJ766303) from 234 nucleotide number to 206 nucleotide number as reverse compliment.

GSP1 primer sequence of *P5βR2* was designed from full length ORF of *P5βR2* (gene bank accession GU062787) from 165 nucleotide number to 138 nucleotide number as reverse compliment. These primers were designed by using Primer3 (http://simgene.com/Primer3) with standard parameters.

For primary PCR, the reaction contents given in Table 3.4 were mixed by vortexing and spun down. For each reaction, 1 µL of GSP1 was added to each tube and 1 µL each DNA library. T100™ Thermal Cycler (BIO-RAD, USA) was used to perform the cycle using the two-step cycle parameters as shown in Table 3.5. 5 µL of the primary PCR products were observed on a 1.5% agarose/EtBr gel using 1X TAE buffer, along with DNA size marker of 1 kb ladder.

3.8.2 Secondary PCR

Primary PCR product was 50 times diluted in 0.5 mL tubes for each sample. Secondary PCR reaction mixture was prepared as shown in Table 3.4. Mixed well by vortexing and briefly spun down the tube in a micro centrifuge. 48 µl of the secondary PCR master mix was added to the tubes. 1 µl of GSP2 and and 1 µL each DNA library were added to each tube. GSP2 primer sequence of 3*β-HSD* was designed from full length ORF of *3β-HSD* (gene bank accession KM406483) from 200 nucleotide number to 171 nucleotide number as reverse compliment. GSP2 primer sequence of *P5βR1* was designed from full length ORF of *P5βR1* (gene bank accession KJ766303) from 228 nucleotide number to 202 nucleotide number as reverse compliment. These primers were designed by using Primer3 (http://simgene.com/Primer3) with standard parameters.

Table 3.4. Primary and secondary PCR reaction mixture for promoter's fragment cloning

Reaction components	**Quantities**
Up (ultra-pure) H_2O	40 µL
10X advantage 2 PCR buffer	5 µL
dNTPs mix (10 mM each)	1 µL
AP1/AP2 (adopter, 10 µM)	1 µL
Advantage 2 polymerase mix (50X)	1 µL
GSP1/GSP2 (gene specific primer1) 10 µM	1 µL
Each DNA library	1 µL
Total volume	**50 µL**

Table 3.5. Primary PCR reaction conditions (Two Step)

First step 7 cycles	**Temperatures**	**Time**
Initial denaturation	94°C	25 sec
Extension	72°C	3 min
Second step 37 cycles		
Denaturation	94°C	25 sec
Annealing	67°C	3 min
Additional cycle		
Extension	67°C	7 min

Table 3.6. Secondary PCR reaction conditions (Two Step)

First step 5 cycles	**Temperatures**	**Time**
Initial denaturation	94°C	25 sec
Extension	72°C	3 min
Second step 20 cycles		
Denaturation	94°C	25 sec
Annealing	67°C	3 min
Additional cycle		
Extension	67°C	7 min

Table 3.7. Ligation of secondary PCR products

Components	**Amount**
Fresh PCR product	3 µL
5X T4 DNA ligase reaction buffer	2 µL
pCR®2.1 vector (25 ng/µL)	1 µL
Water	To a total volume of 9 µL
ExpressLink™ T4 DNA ligase (5 units)	1 µL
Final volume	**10 µL**

GSP2 primer sequence of *P5βR2* was designed from full length ORF of *P5βR2* (gene bank accession GU062787) from 158 nucleotide number to 132 nucleotide number as reverse compliment.

The primer was designed by using Primer3 (http://simgene.com/Primer3) with standard parameters. GSP2 primers are shown in Table 3.3. T100™ Thermal Cycler (BIO-RAD, USA) was used to perform the cycle using the two-step cycle. Parameters as shown in Table 3.6. 5 µL of the secondary PCR products on a 1.5% agarose stained with EtBr was analyzed.

3.9 Subcloning of promoter fragments to cloning vector

For sequencing of promoter fragments, PCR products were ligated into cloning vector pCR2.1 following the instructions of TA Cloning ® Kit manufacturer (Thermo Fisher Scientific, Massachusetts, USA). Ligated product was transformed into competent cells of Top10 *E. coli* by heat shock method. The cells were spread on LB media (Tryptone 10g/L, NaCl 10 g/L and yeast extract 5 g/L, agar 2 g/L, pH 7.0) plates containing 50 µg/mL kanamycin and incubated at 37ºC overnight. Single colonies (5-8) were selected and cultured in liquid LB medium containing 50 µg/mL kanamycin. Plasmid was isolated using a plasmid isolation kit (Thermo Fisher Scientific, Massachusetts, USA). Again PCR was performed (Table 3.8 and Table 3.9) to confirm the presence of promoter fragments into the plasmids by using universal primers M13 (Table 3.10).

After verification, plasmids were sent to a company for sequencing (Prizma lab., Ankara). The promoter fragments were sequenced by Sanger sequencing method.

3.10 Analyses of promoter's sequence

Promoter sequences were analyses by online tool PlantCare (Lescot et al. 2002).

Table 3.8. PCR reaction mixture for cloning of promoters from cloning vectors or colony PCR

PCR reaction components	**Quantity**
5X phusion HF buffer	4 µL
10 mM dNTPs	0.4 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
Phusion Taq DNA polymerase (1.0 units/50 µl PCR)	0.2 µL
Nuclease-free H_2O	12.4
Template DNA/colony mixture (300 ng/µL)	1 µL
Total volume	**20 µL**

Table 3.9. PCR program for cloning of promoters from cloning vectors or colony PCR

Conditions	**Temperatures**	**Time**
Initial denaturation	98°C	30 sec
Denaturation	98°C	10 sec
Annealing	64°C } 30 cycles	30 sec
Extension	72°C	30 sec
Final extension	72°C	10 min
Incubation	4°C	-

Table 3.10. Universal PCR primers for colony PCR

Universal M13 forward primer	5’-GTAAAACGACGGCCAG-3’
Universal M13 reverse primer	5’-CAGGAAACAGCTATGAC-3’

3.11 Isolation of promoters from cloning vectors

Promoters were cloned from cloning vectors (pCR2.1) using promoter specific primers containing restriction enzymes sites. To clone promoter's fragments of *3β-HSD, P5βR1* and *P5βR2* primers with restrictions sites (shown underline) were used (Table 3.11). Sequences with underline shows *Eco*RI and *Nco*I sites. Primers were designed based on promoter sequences using Primer3 (http://simgene.com/Primer3) with standard parameters. Restrictions sites are the same as mentioned above. Reaction mixture for one sample contained as shown in Table 3.7 following the manufacturer instructions (New England BioLabs, Massachusetts, USA). PCR reaction mixture is shown in Table 3.8. Cycling conditions were followed as shown in Table 3.9. The products were confirmed on 1% agarose gel stained with EtBr.

3.12 Digestion of PCR products and subcloning into pCambia 1381

PCR products and pCAmbia 1381 were digested with *Eco*RI and *Nco*I enzymes simultaneously for 1 h. The reaction mixtures were prepared as shown in Tables 3.12 and 3.13. The digested products were run on 1% agarose gel stained with EtBr. Bands were cut with sharp razor and purified according to manufacturer instructions of NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). The purified products were mixed for ligation reaction and placed at 16ºC overnight. Ligation reaction components were mixed as shown in Table 3.14.

3.13 Transformation of ligation products into *Agrobacterium tumefaciens*

Ligation products (pCambia1381+promoter fragment) were transformed into competent cells of *Agrobacterium tumefaciens* strain C58C1. Transformation was performed by heat shock method. 4 µL ligation product was added into the competent cells and incubated on ice for 30 minutes. Then, cells were incubated in 80ºC for 2 minutes. The cells were placed at 37ºC for 5 minutes. Afterward, cells were incubated in ice for few minutes and added SOC media 250 µL. Cells were shaken at 28ºC for 2.5 h at 175 RPM in a shaker. Cells were pelleted and spread on

LB agar media (Table 3.14) plates containing 50 µg/mL kanamycin. Then, cells were placed at 28ºC for two days to obtain colonies.

Table 3.11. List of primers for cloning of promoters from cloning vectors

Primers	**Sequences (5' to 3')**	**Tm**
3β-HSD pro-F 3β-HSD pro-R	5'-GAATTCCACGAAACGCTCGGTTTCC-3' 5'-CCATGGACGGGAGGAAACTTCGCG-3'	60°C
P5βR1 pro-F P5βR1 pro-R	5'-GAATTCAAATGACAACCGTCTAACCGTC-3' 5'-CCATGGGATTTGTGATGTTGGTAGAGGGG-3'	60°C
P5βR2 pro-F P5βR2 pro-R	5'-GAATTCGGTCTGGCTGAAACTCTATCGA-3' 5'-CCATGGCTGGCAGTGTAAGCCGTTC-3	60°C

Table 3.12. Reaction mixture for restriction digestion of PCR products

Restriction digestion reaction components	**Quantity**
*Eco*RI-HF (10 units)	1 µL
*Nco*I-HF (10 units)	1 µL
PCR products (1µg)	20 µL
10X cutsmart buffer	5 µL
Nuclease-free H_2O	23
Total volume	**50 µL**

Table 3.13. Reaction mixture for restriction digestion of pCambia 1381

Restriction digestion reaction components	**Quantity**
*Eco*RI-HF (10 units)	1 µL
*Nco*I-HF (10 units)	1 µL
pCambia 1381 (1µg)	3.33 µL
10X cutsmart buffer	5 µL
Nuclease-free H_2O	39.67 µL
Total volume	**50 µL**

Table 3.14. Ligation of PCR products and pCambia 1381

Components	**Quantity**
EcoRI-HF & *NcoI*-HF PCR purified PCR products (200 ng)	5 µL
5X T4 DNA ligase reaction buffer	2 µL
*Eco*RI-HF & *Nco*I-HF purified pCambia 1381 (300 ng)	2 µL
ExpressLink™ T4 DNA ligase (5 units)	1 µL
Final volume	**10 L**

3.14 Plant growth and transformation of the *3β-HSD, 5βPOR1* and *5βPOR2* promoters into *Arabidopsis thaliana*

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0) plants were grown in peat soil at 25°C under a 16-h-light/8-h-dark cycle. *Agrobacterium tumefaciens* (strain C58C1) mediated transformation of Arabidopsis plants was accomplished using the floral dip protocol (Clough and Bent, 1998). Briefly, healthy Arabidopsis seedlings were grown using peat media until flowering started. Water was supplied once a week with ½ MS media. First bolt was clipped to enhance flowering and after 4-6 days floral dip transformation was performed. *Agrobacterium* strain C58C1 harboring binary vector pCambia1381 containing promoter fragments of *3β-HSD*, *P5βR1* and *P5βR2* were grown on LB media plates containing 50 µg/mL kanamycin. Single colonies were cultured in 5 mL LB media containing 50 µg/mL kanamycin at 28ºC with 175 RPM in a shaker. Overnight bacterial culture was spun down and resuspended in 5% sucrose solution to attain OD_{600}=0.8. Total bacterial culture volume was around 350-400 mL. Silwett L-77 (500 µL/L) was added to the culture before floral dip transformation. Arabidopsis flowers were dipped for 5 seconds and covered with polythene bags to attain high humidity. After 24 h, bags were removed and plants were grown normally until mature seeds were harvested.

T1 generation seeds were selected for resistance to hygromycin. Seeds were surface sterilized with a solution composed of 20% (v/v) commercial bleach and 0.04% (v/v) Tween 20 for 15 min, then rinsed five times with sterile distilled water. Then the seeds were placed on one-half-concentrated Murashige and Skoog (Murashige and Skoog 1962) medium solidified with 0.8% (w/v) agar and supplemented with 25 µg/mL hygromycin. Seeds were placed at 4°C for 48 h in darkness, then germinated. Plants were grown in a growth chamber at 25°C under a 16-h-light/8-h-dark cycle. At least 10 independent transgenic lines were obtained for the construct and transgenes were transferred to sterile peat media for further growth to get T2 seeds development.

3.14.1 Activity of promoter fragment of *3β-HSD* during growth and development in Arabidopsis

Surface sterilized T_2 seeds of promoter fragment of *3β-HSD* were grown on ½ MS media containing 25 µg/mL hygromycin as mentioned above. Seedlings with developing root were selected at the 2, 6 and 20 days' interval and GUS histochemical staining was performed.

3.14.2 Abiotic stress treatments for promoter fragment of *3β-HSD*

For the stress studies, two-week old T_2 Arabidopsis seedlings of promoter fragment of *3β-HSD* were subjected to salt, sucrose and mannitol stress. For salt stress, 150 mM NaCl was added to agar only (no MS media). For sucrose, 3% sucrose was added only to agar (no MS media). For drought stress, 2% mannitol was used. The above-mentioned media were prepared by adjusting pH 5.8 with KOH/HCl and autoclaved at 121ºC for 15 minutes. The seedlings were placed on the stress media plates including control plate containing MS media and 3% sucrose. After 24 h treatment the seedlings were harvested and GUS activity was observed using GUS histochemical staining.

3.14.3 Activity of promoter fragment of *5βPOR1* and*5βPOR2* during growth and development in *Arabidopsis thaliana*

T_2 seedlings of promoter fragments of the *5βPOR1 and 5βPOR2* were grown on ½ MS media (DUCHEFA BIOCHEMIE, The Netherlands) containing 25 µg/mL hygromycin as mentioned above. Seedlings with developing root were selected at the 5, 10 and 15 days' interval and GUS histochemical staining was performed.

3.15 GUS Histochemical staining

For histochemical GUS expression analysis, the transgenic Arabidopsis seedlings were incubated in the GUS staining solution (0.2 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.0, and 2 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid) at 37°C for 24 h, followed by washing in 70% ethanol according to a published method

(Jefferson et al., 1987). After complete removal of chlorophyll by series of ethanol 10%, 30%, 50% and 70% each 30 minutes and achieving transparency, the seedlings were observed and photographed were acquired with a Leica DM1000 LED microscope.

4. RESULTS AND DISCUSSIONS

4.1 Cloning of promoter fragment of *3β-HSD*

Four gDNA libraries were PCR amplified. Promoter fragment of *3βHSD* was isolated from gDNA library 4 (DL4) which was produced by *Stu*I enzyme digested library used for *3β-HSD* promoter cloning (Figure 4.1).

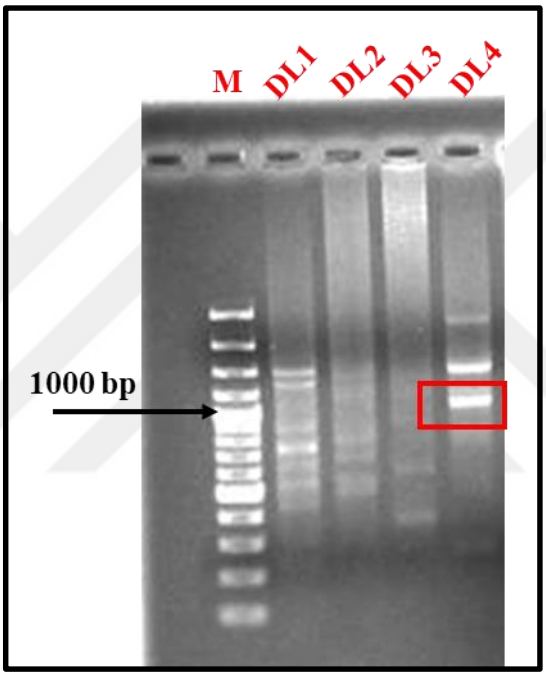

Figure 4.1. Promoter cloning of the *3βHSD*. *Dra* I (DL1), *EcoR* V (DL2), *Pvu* II (DL3) and *Stu* I (DL4)

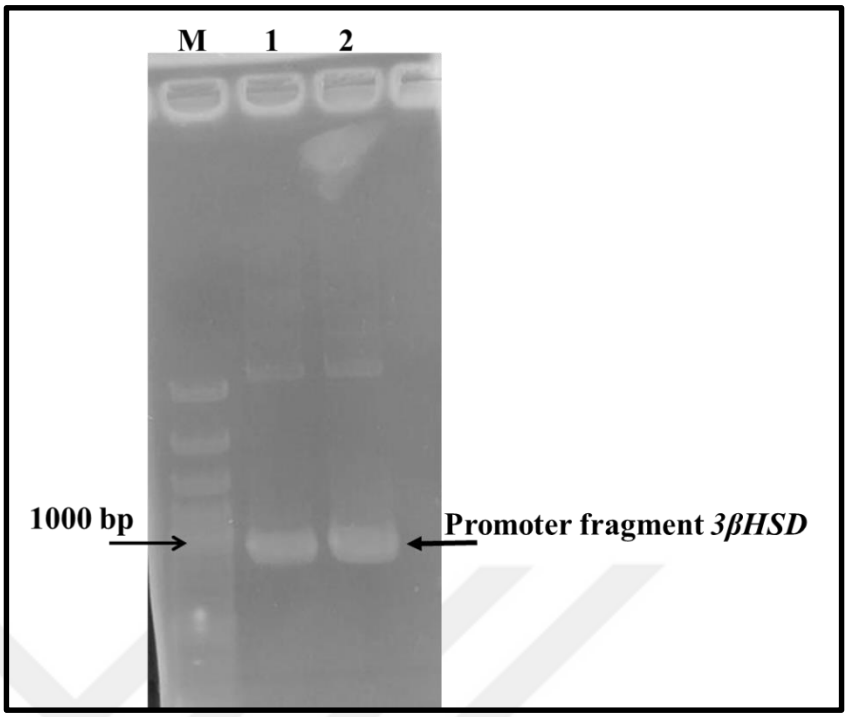

Figure 4.2. Colony PCR for confirmation of promoter fragment in pCR2.1 cloning vector

>Promoter 3β-HSD
CACGAAACGCTCGGTTTCCATTCGGTTGTAAGGTAGCCACCCCGCTATTAAGGATATGCAGAAAGAAATAA
TTGAAAAAGAGCTCCAGTATAAAGATCTTCATTAGTGCGTAACCCGATTGTATACCACCACTGATAAACAC
CAGAATAAGCGATATTCACTGGGCCAAGAGCACCCCCTCGAGTAAAAGCTTCCACGGCCGGTTGACCAAAA
TGAGGATCCCAAATTGCATGAGCAATAGGTCTTACATGTAAAGGGTCCTGCACCCATGATTCAAAATTTCC
TTGCCAAGCTACATGAAAGAGATTTCCGGAAGTCCACAGAAAAATTATTGCTAATTGACCGAAGTGAGAAG
CAAAAATATTCTGATAAAGACGTTCCTCAGTAATATCATCATGACTTTCGAAGTCATGTGCGGTAGCAATA
CCAAACCAAATACGACGAGTAGTGGGGTCCTGAGCTAAGCCTTGGCTAAACCTTGGAAATCTTAATGCCAT
AATGCCTTTCAAATCCTCCTAGCCATTATCCTACTGCAATAATTCTTGCTAAGAAGAACGCCCATGTTGTG
GCAATTCCACCCAGAAGGTAATGGGTTACTCCTACAGCACGTCCTTGTACAATGCTCAAGGCTCTCGGCTGA
GTAGCAGGAGCAACTTTTAATTTATTATGAGCCCAAACGATGGATTCAATAAGTTCTTGCCAATAACCACGT
CCACTGAATAGAAACATTAAACTAAAAGCCCATACAAAATGAGCGCCTAGGAAAAAAAAGGCCATATGCAGATA
ATGAAGAACCATAAGACTGAATTACCTGGGATGCCTGTGCCCATAAGAAATCCCGGAGCCACCCATTAATAGT
AATAGAACTCTGCGCGAAGTTTCCTCCCGT

Figure 4.3. Sequence (888 bp) of promoter fragment of the *3β-HSD*

Table 4.1. *Cis*-elements of the *3B-HSDpromoter*

Site Name	Organism	Position	Matrix score	sequence	Fucntion
AAGAA-motif	*Avena sativa*	60	7	GAAAGAA	*cis*-acting element conferring high transcription levels
AE-box	*Arabidopsis thaliana*	721	8	AGAAACAT	Part of module for light response
ARE	*Zea mays*	428	6	TGGTTT	*cis*-acting regulatory element essential for the anaerobic induction
Box 4	*Petroselinum crispum*	849	7	ATTAAT	part of a conserved DNA module involved in light responsiveness
Box I	*Pisum sativum*	503	7	TTTCAAA	light responsive element
Box-W1	*Petroselinum crispum*	203, 338	6	TTGACC	fungal elicitor responsive element
CAAT-box	*Glycine max* *Hordeum vulgare* *Brassica rapa* *Glycine max* *Hordeum vulgare* *Glycine max* *Hordeum vulgare* *Brassica rapa*	69 70, 117 222 224 225, 235, 330 336 337, 421 432, 506 533 567	5 4 5 5 4 5 4 5 4	CAATT CAAT CAAAT CAATT CAAT CAATT CAAT CAAAT CAAT	common *cis*-acting element in promoter and enhancer regions

Table 4.1 (Continued)

	Hordeum vulgare *Arabidopsis thaliana* *Glycine max* *Hordeum vulgare* *Arabidopsis thaliana* *Hordeum vulgare*	569 617, 686 699 700	6 5 4 5 4	GGCAAT CAATT CAAT CCAAT CAAT	
G-box	*Glycine max*	605, 707	6	CACGTC	*cis*-acting regulatory element involved in light responsiveness
LAMP-element	*Pisum sativum*	366	8	CTTTATCA	part of a light responsive element
MBS	*Zea mays*	339	6	CGGTCA	MYB Binding Site
MNF1	*Zea mays*	821	7	GTGCCC(A/T)(A/T)	light responsive element
Skn-1_motif	*Oryza sativa*	395, 407	5	GTCAT	*cis*-acting regulatory element required for endosperm expression
Sp1	*Zea mays*	36, 843, 574, 880	5.5 5.5 5.5 5	CC(G/A)CCC	Light responsive element
TATA-box	*Glycine max* *Lycopersicon esculentum* *Glycine max* *Helianthus annuus*	45 654, 184 851 119	5 5 5 5	TAATA TTTTA TAATA TATACA	core promoter element around -30 of transcription start

Table 4.1 (Continued)

	Lycopersicon esculentum *Glycine max* *Arabidopsis thaliana* *Glycine max* *Arabidopsis thaliana* *Oryza sativa*	734 385, 857 88 662 121 744	5 5 6 5 4 7	TTTTA TAATA TATAAAA TAATA TATAAAA TACAAAA	
TCT-motif	*Arabidopsis thaliana*	242	6	TCTTAC	Part of light responsive element
Unnamed_3	*Zea mays*	193, 706	5	CGTGG	-
Unnamed_4	*Petroselinum hortense*	82, 839, 596, 881, 512, 646	4	CTCC	-
Unnamed_6	*Zea mays*	88	10	taTAAATATct	-
W box	*Arabidopsis thaliana*	203, 338	6	TTGACC	-
WUN-motif	*Brassica oleracea*	276	9	AAATTTCCT	Wound response element
As-2-box	*Nicotiana tabacum*	781	9	GATAatGATG	Involved in shoot specific expression and light responsiveness
Box S	*Arabidopsis thaliana*	34, 841	7	AGCCACC	-
Chs-CMA2a	*Hordeum vulgare*	568	8	GCAATTCC	Part of a light responsive element

The PCR amplified fragment as shown in Figure 4.1 was cloned into cloning vector and colony PCR was performed (Figure 4.2). A fragment of about 1 Kb was obtained. The positive colony was cultured in LB liquid media overnight and plasmid was isolated. The plasmid containing promoter fragment of *3β-HSD* was sequenced and promoter sequence was analyzed.

4.1.1 *Cis*-acting elements in promoter fragment of *3β-HSD*

The sequence (888 bp) analysis of the *3β-HSD* promoter (Figure 4.3) revealed the presence *cis*-acting element for high transcription level, part of module for light, anaerobic induction, conserved DNA module involved in light responsiveness, fungal elicitor responsive element, common *cis*-acting element in promoter and enhancer regions, *cis*-acting regulatory element involved in light responsiveness, part of a light responsive element, MYB binding site, light responsive element, *cis*-acting regulatory element required for endosperm expression, light responsive element, core promoter element around -30 of transcription start, part of light responsive element, wound response element, involved in shoot specific expression and light responsiveness and part of a light responsive elements were found in promoter fragment of *3β-HSD*. Unknown function *cis*-acting elements like unnamed_3, unnamed_4 and unnamed_6 were also found (Table. 4.1). Promoter sequence of *3βHSD* revealed that it has novel and important *cis*-elements for biotic and abiotic stress factors such as fungal elicitors or wound response. In addition, it also contains light response elements and some unknown (uncharacterized) *cis*-elements.

Light response elements or modules (AE-box, Box 4, Box I, G-box, LAMP-element, MNF1, Sp1, TCT- motif, As-2-box, Chs-CMA2a) were found in *3β-HSD* promoter. These elements are usually present in the genes which are light regulated and induce gene expression in the presence of light (Arguello-Astorga and Herrera-Estrella 1998). The I-Box are involved in light response and also circadian clock response (Borello, Ceccarelli, and Giuliano 1993). The G-Boxes were found in the promoters of genes which regulated due to light or other stimuli responses (Schulze-Lefert et al. 1989, Block et al. 1990, Schindler et al. 1992, Lopez-Ochoa et al. 2007). AE boxes alone cannot work on the same promoters for light responses and work in combination with Gap boxes (Park, Kwon, and Shih 1996). LAMP-element has been found strongly conserved in phytochrome-regulated promoters (Grob and Stuber

1987). Box-W1 (fungal elicitor responsive element) was found in *WRKY1* gene promoter for the first time in parsley. Treatment of parsley cells with oligopeptide elicitor Pep25, the expression of promoter was highly induced in the presence of W1 (Rushton et al. 1996). Two fungal elicitor *cis*-elements were found in *3β-HSD* promoter. ARE is a putative binding site for a Myb found in the promoter of maize glycolytic glyceraldehyde-3-phospate dehydrogenase 4 (GapC4) which is essential for anaerobic induction (Geffers et al. 2001). MBS is a MYB binding site involved in drought-inducibility (Abe et al. 1997) and one element is present in *3β-HSD* promoter. Skn-1_motif a *cis*-acting regulatory element required for endosperm expression (Takaiwa et al. 1991) also one element is present in *3β-HSD* promoter. One wound response element (WUN-motif) (Pastuglia et al. 1997) present in this promoter.

4.1.2 Development of transgenic Arabidopsis plants carrying *3β-HSDpromoter*::GUS

The *3β-HSD*promoter was subcloned into pCambia 1381 in *Eco*RI and *Nco*I restriction sites (Figure 4.4) and then Arabidopsis transformed plants with promoter fragment of *3β-HSDpro*::GUS were obtained (Figure 4.5), however, transformation efficiency (1.05%) (Figure 4.5. F, G) seems to be low as compared to previously published reports (Clough and Bent 1998). In spite of low transformation efficiency, enough amount of transgenic seeds can be obtained if transformation of same plant should be repeated at least 2 time at one-week interval.

The plants are weaker than non-transformed plants due to possibly antibiotic stress. Therefore, the selected transformants plants need more time to achieve enough growth for further PCR based confirmation. 3-4 weeks old Arabidopsis plants ready to transform by floral dip transformation (A). Inverted Arabidopsis plants to full dip floral parts into Agrobacterium suspension culture and held for 5 seconds (B). After transformation place aside the plants on towel tissue paper to remove excessive amount of *Agrobacteria* (C). Cover polythene bag to maintain high humidity over 24 h period (D). After one month later seeds were harvested and surface sterilized (E). These seeds were germinated on ½ MS plates containing 25 mg/L hygromycin. Transgenic seedlings grew to second leaf stage and non-transgenic remained with cotyledons only (F). Transgenic seedlings were selected and transferred to sterile

peat media. Upper panel: transgenic Arabidopsis seedlings growing 2 weeks and lower panel: 4 weeks stage since transferred to peat media (G).

These plants after transgene confirmation were grown to produce until T2 seeds. Then, T2 seeds were grown on selection media and used for promoter characterization which were scored and visualized by GUS histochemical analysis.

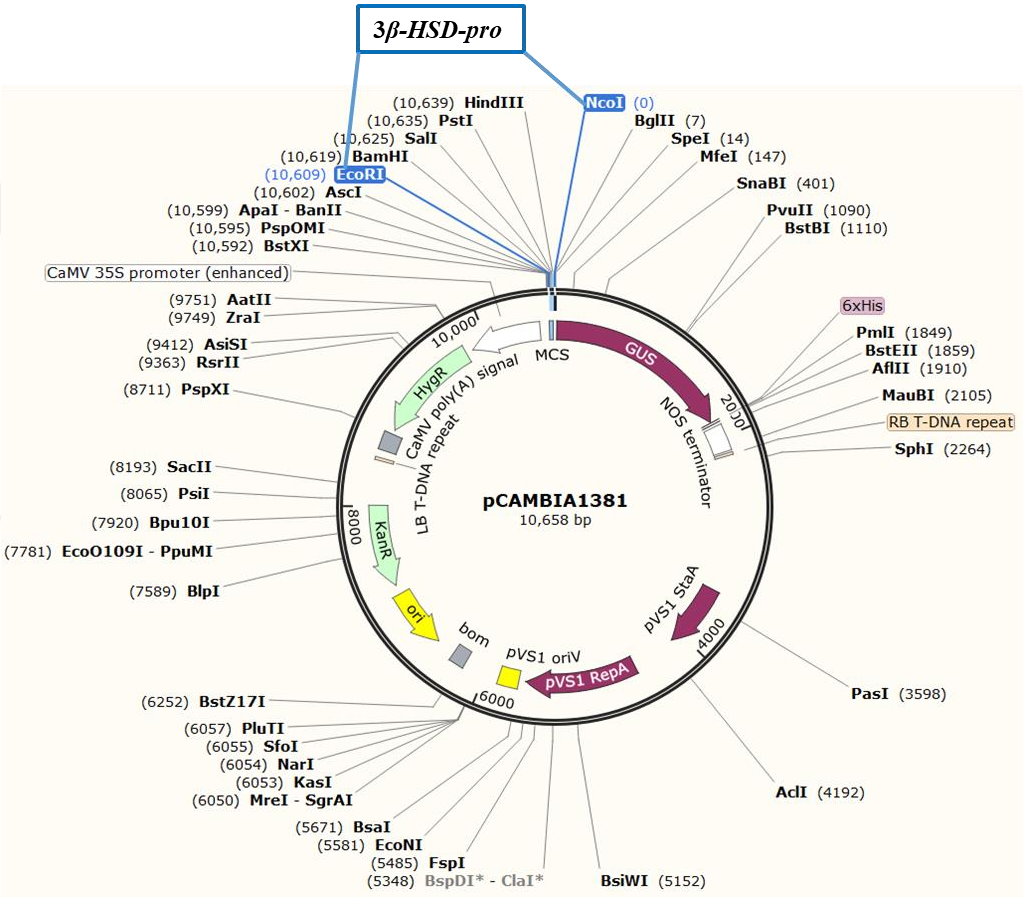

Figure 4.4. Insertion of *3β-HSDpromoter* into pCambia 1381 for the development of *3β-HSDpromoter*::GUS construct for Arabidopsis transformation.

Figure 4.5. Floral dip transformation of Arabidopsis plants for development of *3β-HSDpromoter*::GUS plants.

Figure 4.5 continued

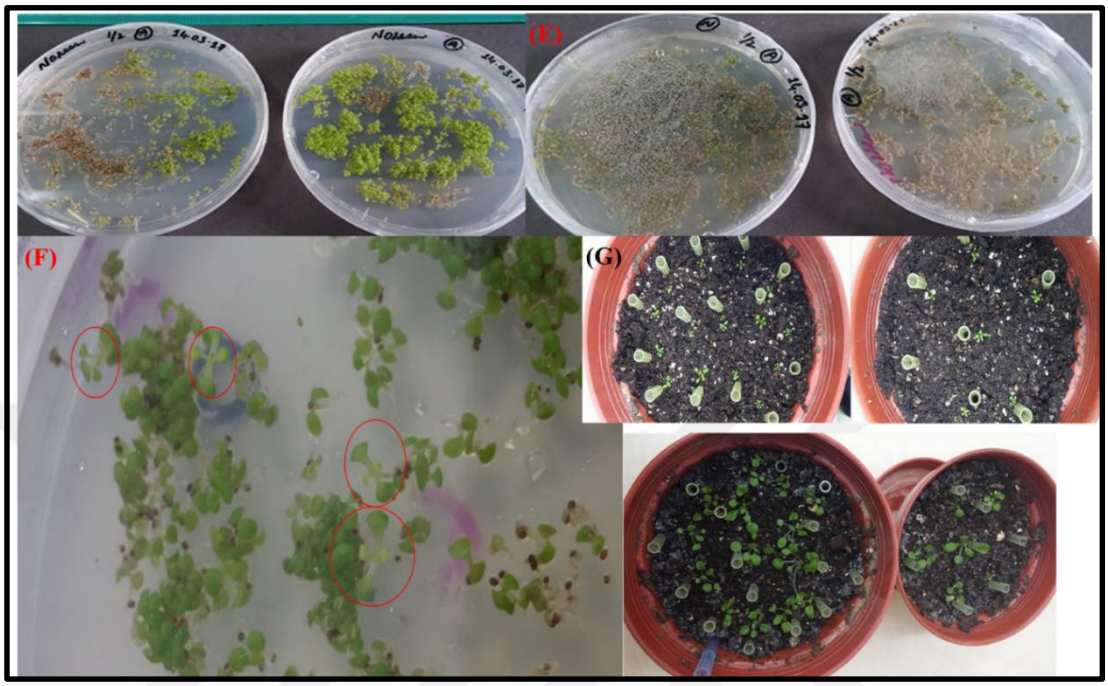

Figure 4.5. T1 transgenic Arabidopsis seed selection on ½ MS media containing 25 mg/L hygromycin and transferred to sterile peat soil.

4.1.3 Activity of *3β-HSDpromoter*::GUS during growth and development stages and abiotic stresses

3β-HSDpromoter expression driven by GUS reporter gene in Arabidopsis T2 plant determined under normal plant growth and development stage GUS activity was observed in 2 days old seedling (Figure 4.6 A). The expression of promoter was confined to cotyledon and to some extent in vascular tissue in hypocotyl region. In root zone expression was just before root tip to the middle of root as indicated by red bracket. The strong expression was found in 6 days old seedling at cotyledonary leaf and in hypocotyl region (Figure 4.6 B). Promoter activity monitored by GUS histochemical staining in the leaves (both young and old), shoot apical meristem, trichome, hydathodes, hypocotyl region and root (Fig. C-G). In mature leaf GUS activity was found in vascular tissues or hydathodes (Fig. 1 C). On the other hand, in young leaves, promoter activity was evident in trichomes (yellow arrow head), hydathodes (purple arrow head), vascular tissues, mid rib (green arrow head) and (SAM) shoot apical meristem (red arrow head) (Figure 4.6 D, E). Further strong GUS activity was observed in leaf primordia (yellow arrow head) and hypocotyl region (red arrow head) (Figure 4.6 F). SAM is the region in the growing shoot containing meristematic cells. The shoot apical meristem contains multipotent stem cells and produces primordia that develop into all the above ground organs of a plant. In root tissues, promoter activity was restricted to xylem tissues (vascular parenchyma, shown as red arrow head), lateral root vascular tissues (yellow arrow head) and root hairs shown by green arrow head (Figure 4.6 G). *AtHSD1promoter* activity was observed in shoot more extensively than in root tissues (Li et al. 2007). 2 days old seedlings expressing *3β-HSDpromoter* activity had a similar fashion of expression (Figure 4.6 A). However, this expression activity was enhanced with the seedling growth and developmental stages of 6 days (Figure 4.6 B) and 20 days seedlings (Figure 4.6 C). The promoter activity was enhanced in 20 days old seedlings in all above ground and root tissues and more intensively in vascular tissues, shoot apical meristem (SAM), hydathodes, trichomes, lateral primary root and root hairs. The promoter activity in apical meristem showed that maybe *3β-HSDpro* play a role in seedling growth and further may also play role in transport of nutrients as it expressed in root hair and root vascular tissues. Under stress conditions, (Figure 4.7) *3β-HSDpromoter* activity was enhanced under sucrose and

2% mannitol treatment, however, downregulated in salt stress conditions. Our findings are consistent with the results of *AtSDR1promoter*::GUS activity where expression was enhanced with 6% mannitol in shoot and root tissues (Cheng et al., 2002)

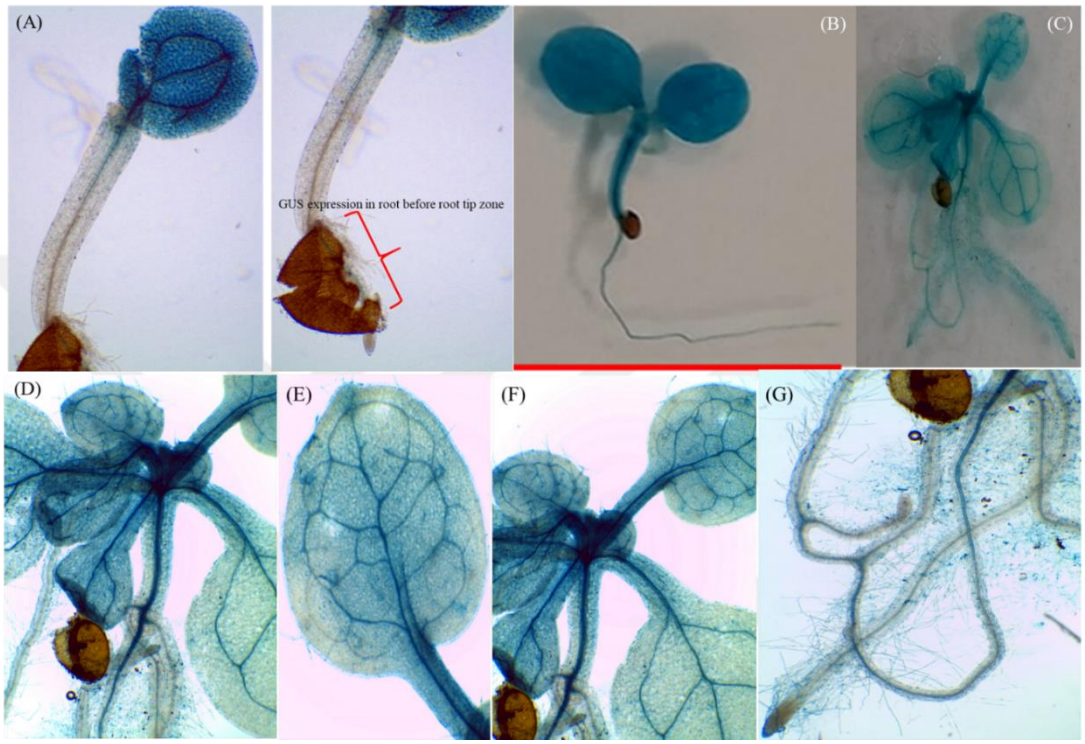

Figure 4.6. *3β-HSDpromoter*::GUS activity in Arabidopsis seedlings during growth and developmental stages. (A) 2 days old seedling, B) 6 days old seedling, C) 20 days old seedling expression in shoot, hypocotyl and root regions; red arrow shows hydathodes aperture, yellowish arrow shows vascular bundle in mature leaf. D) red arrow shows shoot apical meristem from where new cells emerge, purple arrow head shows hydathodes and yellow arrow head shows trichomes, green arrow head shows midrib of young leaf. E) young leaf. F) leaf primordia shown by yellow arrow head and red arrow head shows hypocotyl region. G) xylem tissues (vascular parenchyma, shown as red arrow head), lateral root vascular tissues (yellow arrow head) and root hairs shown by green arrow head.

Under salinity stress (150 mM NaCl) expression of promoter was decreased in fully expanded leaves compared to control (CK) (Figure 4.8). In sucrose application (3%) expression was slightly higher in fully expanded leaves and absent in root apical meristem and elongation zone. Under drought stress governed by mannitol application GUS activity was increased in shoot and root more than CK. Salinity stress decreased promoter activity in cotyledon tissues but not in vascular or SAM compared to control seedlings. The strong expression of the *3β-HSDpromoter* under the growth stages and abiotic stress shows that the *3β-HSD* can regulate under the both conditions.

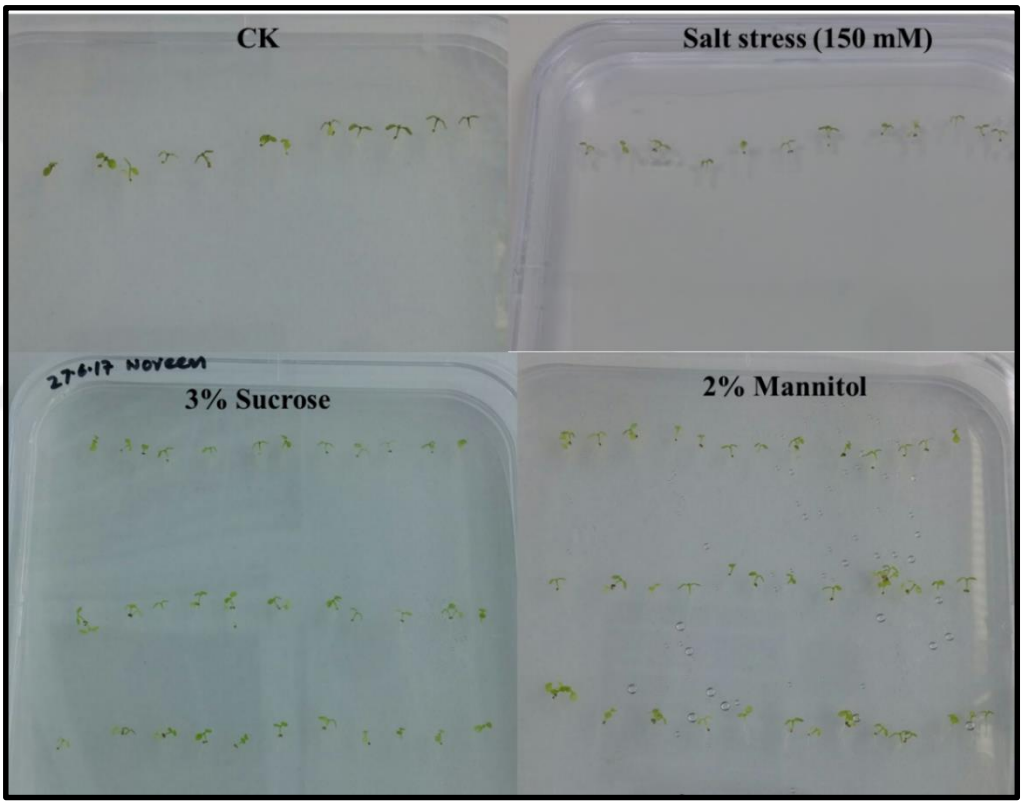

Figure 4.7. T3 Arabidopsis seedlings of 3*β-HSDpromoter*::GUS under different treatments.

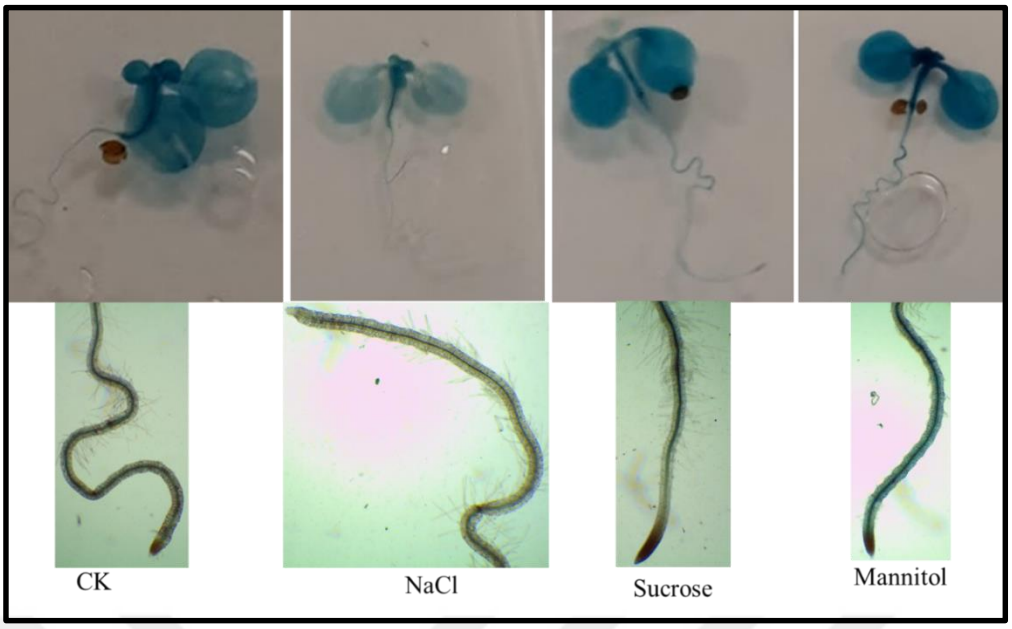

Figure 4.8. *3β-HSDpromoter*::GUS activity under abiotic stress conditions. 15 days old seedlings treated as described in Materials and Methods section. The seedlings were GUS stained after treatment and photographed. Upper panel is whole seedling while lower panel shows root portion.

4.2 Cloning and analysis of the *5βPOR1* promoter

The promoter *5βPOR1* was cloned from genomic DNA library digested with *Dra*I restriction enzyme (Figure 4.9).

The sequence analysis of the *5βPOR1* promoter (764 bp) (Figure 4.10) revealed the presence of *cis*-acting element conferring high transcription levels, *cis*-acting element involved in the abscisic acid responsiveness, anaerobic induction, elicitor-mediated activation, light responsive module, light responsive element, fungal elicitor responsive element, elicitor-responsive element, *cis*-acting element involved in heat stress responsiveness, MYB binding site, *cis*-acting regulatory element required for endosperm expression and *cis*-acting regulatory element involved in circadian control and also core elements of promoter such as CAAT and TATA-box (Table 4.2).

In the *5βPOR1promoter* the *cis*-acting elements such as elicitor-responsive element, light responsive module, light responsive element, fungal elicitor responsive element, anaerobic induction, MYB binding site, *cis*-acting regulatory element required for endosperm expression are the same as mentioned under the section *Cis*-acting elements in promoter fragment of *3β-HSD*. However, few novel response elements were found in the *5βPOR1promoter*. Abscisic acid responsiveness (Zhu 2002), *cis*-acting regulatory element involved in circadian control (Harmer et al. 2000) and *cis*-acting element involved in heat stress responsiveness (Bharti et al. 2000) are the novel elements not found in *3β-HSDpromoter***.** The presence of abscisic acid and heat stress responsiveness elements showed that the *5βPOR1* can be regulated under stress conditions such as salinity, drought and heat stresses. Since ABA mediates environmental responses such as cold, drought and salinity (Zhang et al. 2005).

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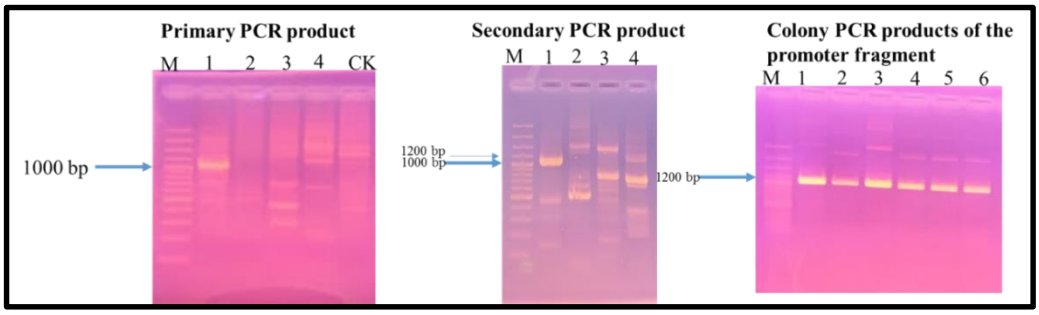

Figure 4.9. Cloning of *5βPOR1* promoter in primary and secondary PCR steps and confirmation of subcloning into cloning vector by colony PCR. In primary and secondary PCR products; Lanes 1: genomic DNA digested with *Dra*I, 2: *Eco*RV 3: *Pvu*II, 4: *Stu*I. The approximate size of promoter is 1100 bp. However, after sequence analysis exon region was removed and final size of the promoter obtained was 763 bp as shown below in the sequence.

>Promoter *5βPOR1*

AAATGACAACCGTCTAACCGTCTGCTAATTATATCACCCGACCTGCGGTCGCCATA
ATTTATTATTTGACCAACCCACTTATGATGGGTCATGTTGATTTTTTATATCAATCCG
CCAAAATTCGACCGTATACACCCTTAATAAAAGTTGTAAAAATCATTTTAATTTCAAGT
TTTTAACTAAATCGATCAAAAGCAAAATTTTAATCATACTAAAATACTGCCAAAAAATG
AAAAAATAGAGATTTTTTTGAATGTTGTTCATTACGTTCAAGAACAATCCACAAAGTTTA
TAACATAGAAAAAAGTTTCAAATGTTATTAATGGTTTCAAAAATTACTTTTTATTTTGTC
TTCGATTGAAACATTTAGAAATTGTAGTCTATGCTTATAATTCTATTTTCTTATTATTTT
ATCACGCTTTAGTCTAATTTTATGTTATTACTTTTGTGTGTAAAAGAAATTCAAGTAAATT
ACACACACATCTGTCCAATTTATAGCAAAATCTATCCGGTCGGTCAACACATCATAATAAAT
AATAATATGTGTAAAAGAAATTTCAAGTAAATTACACACACATCTGTCCAATTTATAGCAAA
ATCTATCCGGTCGGTCAACACATCATAATAAATAATAATAAGTCTGGTTCCTTTCCTTCTCT
TCCACCTGTCACTACCAATCACCACACAAAACGAGATTCAGGCGCTAATAAAGCTCGCAGCT
ATCTCAGATCTTTATCAGTTCCCCTCTACCAACATCACAAATC

Figure 4.10. Sequence (764 bp) of promoter fragment of the 5*βPOR1*

Table 4.2. *Cis*-elements of the *5βPOR1promoter*

Site name	Organism	position	Matrix score	Sequence	Function
5UTR Py-rich stretch	*Lycopersicon esculentum*	649	9	TTTCTTCTCT	*cis*-acting element conferring high transcription levels
ABRE	*Arabidopsis thaliana*	123	7	TACGGTC	*cis*-acting element involved in the abscisic acid responsiveness
AC-II	*Phaseolus vulgaris*	64	9	(C/T)T(T/C)(C/T)(A/C)(A/C)C(A/C)A(A/C)C(C/A)(C/A)C	-
ARE	*Zea mays*	323	6	TGGTTT	*cis*-acting regulatory element essential for the anaerobic induction
AT-rich sequence	*Pisum sativum*	212	9	TAAAATACT	element for maximal elicitor-mediated activation (2copies)
AT1-motif	*Solanum tuberosum*	333	13	AATTATTTTTTATT	part of a light responsive module
Box 4	*Petroselinum crispum*	318	6	ATTAAT	part of a conserved DNA module involved in light responsiveness
Box I	*Pisum sativum*	307	7	TTTCAAA	light responsive element
Box I	*Pisum sativum*	326	7	TTTCAAA	light responsive element
Box-W1	*Petroselinum crispum*	65	6	TTGACC	fungal elicitor responsive element
Box-W1	*Petroselinum crispum*	609	6	TTGACC	fungal elicitor responsive element
Box-W1	*Petroselinum crispum*	514	6	TTGACC	fungal elicitor responsive element
CAAT-box	*Brassica rapa*	63	5	CAAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-	*Glycine*	488	5	CAATT	common *cis*-acting

Table 4.2 (Continued)

box	*max*				element in promoter and enhancer regions
CAAT-box	*Hordeum vulgare*	356	4	CAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Hordeum vulgare*	674	4	CAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Hordeum vulgare*	276	4	CAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Glycine max*	583	5	CAATT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Hordeum vulgare*	372	4	CAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Brassica rapa*	758	5	CAAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Hordeum vulgare*	107	4	CAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Arabidopsis thaliana*	582	5	CCAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Glycine max*	371	5	CAATT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Arabidopsis thaliana*	673	5	CCAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Brassica rapa*	310	5	CAAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Arabidopsis thaliana*	487	5	CCAAT	common cis-acting element in promoter and enhancer regions
EIRE	*Nicotiana tabacum*	120	7	TTCGACC	elicitor-responsive element
G-box	*Glycine max*	659	12	tcCACGTGTCACT	*cis*-acting regulatory element involved in light responsiveness
GT1-motif	*Solanum tuberosum*	277	8	AATCCACA	light responsive element
HSE	*Brassica oleracea*	240	9	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness
HSE	*Brassica oleracea*	549	9	AAAAAATTTC	*cis*-acting element involved in heat

Table 4.2 (Continued)

					stress responsiveness
HSE	*Brassica oleracea*	301	9	AAAAAA TTTC	*cis*-acting element involved in heat stress responsiveness
LAMP-element	*Pisum sativum*	730	8	CTTTAT CA	part of a light responsive element
MBS	*Zea mays*	513	6	CGGTCA	MYB Binding Site
MBS	*Zea mays*	608	6	CGGTCA	MYB Binding Site
Skn-1_motif	*Oryza sativa*	2	5	GTCAT	*cis*-acting regulatory element required for endosperm expression
Skn-1_motif	*Oryza sativa*	87	5	GTCAT	*cis*-acting regulatory element required for endosperm expression
TATA-box	*Brassica napus*	27	6	ATTATA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	28	5	TATAA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	29	4	TATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	59	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	99	7	TATAAA A	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	100	6	TATAAA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	101	5	TATAA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	102	9	taTATAA Atc	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	128	4	TATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	138	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Lycopersicon esculentum*	141	5	TTTTA	core promoter element around -30 of transcription start

Table 4.2 (Continued)

TATA-box	*Lycopersicon esculentum*	150	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*Lycopersicon esculentum*	159	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*Lycopersicon esculentum*	173	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*Lycopersicon esculentum*	200	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*Lycopersicon esculentum*	212	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	288	6	TATAAA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	289	5	TATAA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	290	4	TATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	317	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Lycopersicon esculentum*	340	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	386	5	TATAA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	387	4	TATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	403	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Lycopersicon esculentum*	408	5	TTTTA	core promoter element around -30 of transcription start
TATA-	*Lycopersi*	429	5	TTTTA	core promoter

Table 4.2 (Continued)

box	*con esculentum*				element around -30 of transcription start
TATA-box	*Glycine max*	437	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Lycopersicon esculentum*	452	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*Antirrhinum majus*	489	8	TATAAATT	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	490	7	TATAAAT	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	491	6	TATAAA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	492	5	TATAA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	493	4	TATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	527	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	534	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	537	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Lycopersicon esculentum*	546	5	TTTTA	core promoter element around -30 of transcription start
TATA-box	*Antirrhinum majus*	584	8	TATAAATT	core promoter element around -30 of transcription start
TATA-box	*Ac*	585	7	TATAAAT	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	586	6	TATAAA	core promoter element around -30 of transcription start
TATA-box	*Arabidopsis thaliana*	587	5	TATAA	core promoter element around -30 of transcription start

Table 4.2 (Continued)

TATA-box	*Arabidopsis thaliana*	588	4	TATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	622	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	629	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	632	5	TAATA	core promoter element around -30 of transcription start
TATA-box	*Glycine max*	704	5	TAATA	core promoter element around -30 of transcription start
TCCACCT-motif	*Petroselinum hortense*	659	7	TCCACCT	-
W box	*Arabidopsis thaliana*	65	6	TTGACC	-
W box	*Arabidopsis thaliana*	609	6	TTGACC	-
W box	*Arabidopsis thaliana*	514	6	TTGACC	-
circadian	*Lycopersicon esculentum*	242	6	CAANNNNATC	*cis*-acting regulatory element involved in circadian control

4.2.1 Development of transgenic Arabidopsis plants carrying *5βPOR1promoter*::GUS

The *5βPOR1promoter* was subcloned into pCambia 1381 in *Eco*RI and *Nco*I restriction sites (Figure 4.11) and transformed into Arabidopsis via floral dip transformation (Figure 4.12). The transgenic seedlings were selected on the ½ MS agar media containing hygromycin, onset of second true leaves (Figure 4.12 C). The T1 transgenic seedlings were transferred to sterile peat media and covered with polythene sheet for acclimatization. The seedlings were grown to seed maturity level and T2 seeds were harvested. The T2 seeds were grown aseptically on ½ MS media containing hygromycin and the seedlings (Figure 4.13) were selected at 5, 10 and 15 days interval and promoter activity was observed.

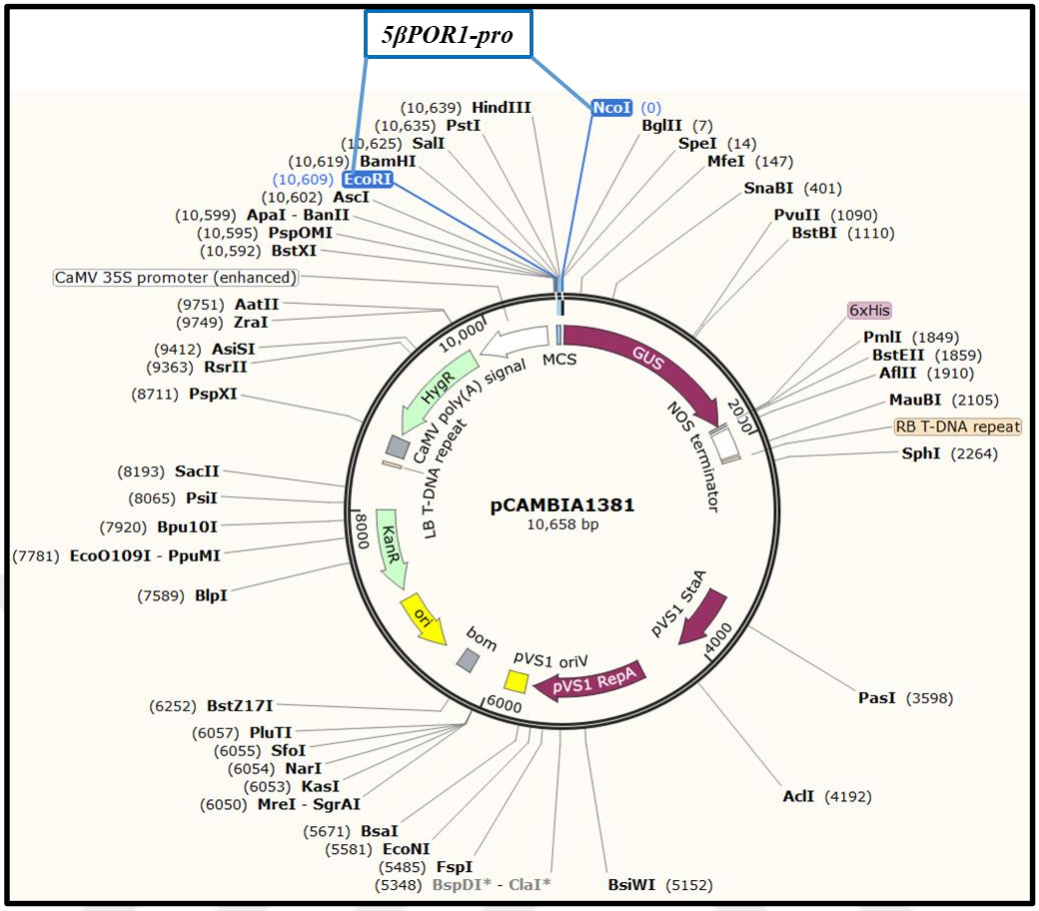

Figure 4.11. Insertion of 5*βPOR1promoter* into pCambia 1381 for the development of 5*βPOR1promoter*::GUS construct for Arabidopsis transformation.

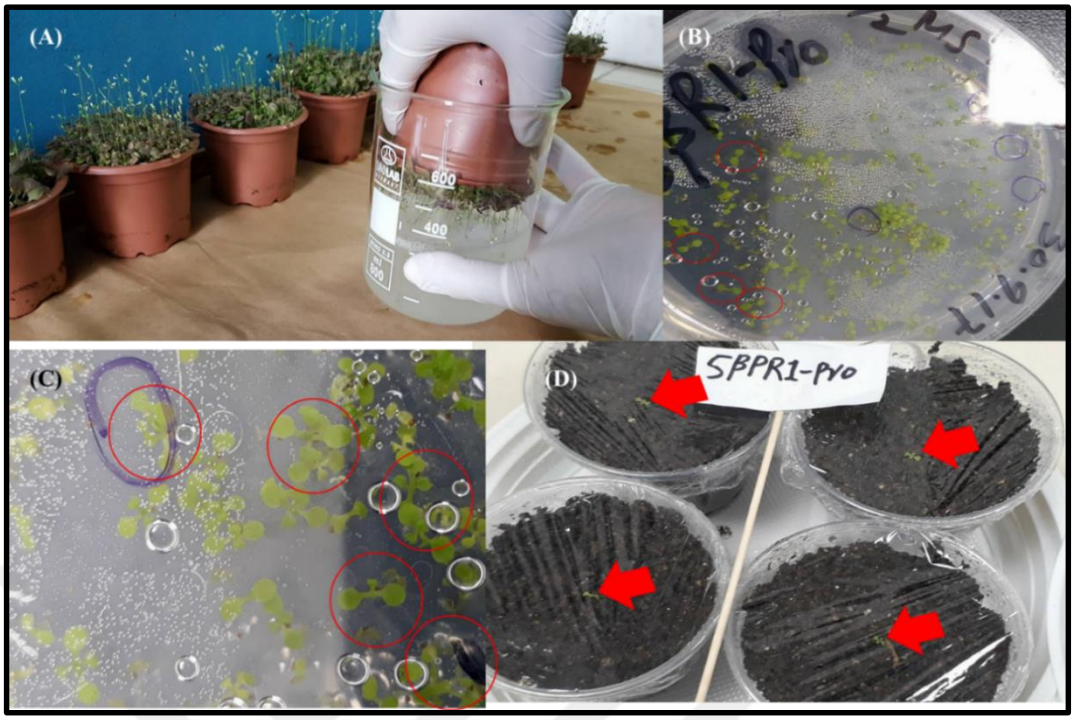

Figure 4.12. Development of transgenic Arabidopsis plants carrying promoter fragment of the *5βPOR1promoter*::GUS. (A) Floral dip transformation of Arabidopsis seedlings. (B) selection of transgenic Arabidopsis seedling on ½MS media containing 25 µg/mL hygromycin. (C) Second leaf emerged from transgenic seedlings and circled with red color. (D) Transgenic Arabidopsis seedlings were transferred to sterile peat media and covered with transparent polythene sheet for acclimatization. Red arrow shows seedlings in the pots.

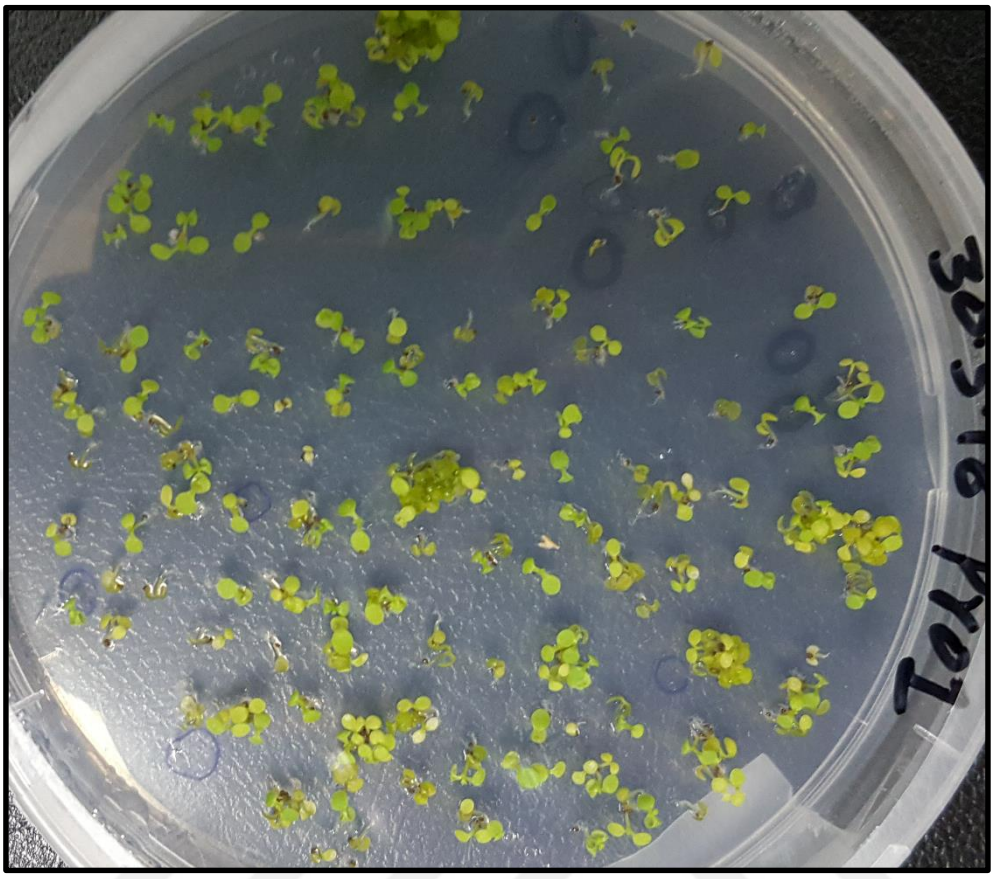

Figure 4.13. Transgenic Arabidopsis T2 seedlings of *5βPOR1promoter*::GUS used for GUS histochemical analysis at 5, 10 and 15 days interval during seedling growth and development stages.

4.2.2 Activity of *5βPOR1promoter*::GUS during growth and development stages

4.2.2.1 Five days old seedlings

Five days old seedling of *5βPOR1promoter*::GUS was subjected to GUS histochemical analysis. Staining revealed that in 5 days old seedlings, strong expression activity of *5βPOR1promoter*::GUS was found in cotyledons (Figure 4.14 A), hypocotyl and root (Figure 4.14 B), root (Figure 4.14 C, D and E), root hairs (Figure 4.13 E) indicated by red arrows head, root but not in root tip (meristem zone, Figure 4.14 C) indicated by red arrow head. Further, the strongest promoter activity was found in vascular tissues (Figure 4.14 A, D) indicated by red and green arrow heads. These results show that *5βPOR1promoter* has the highest expression activity at early seedling development stage in all tissues of Arabidopsis.

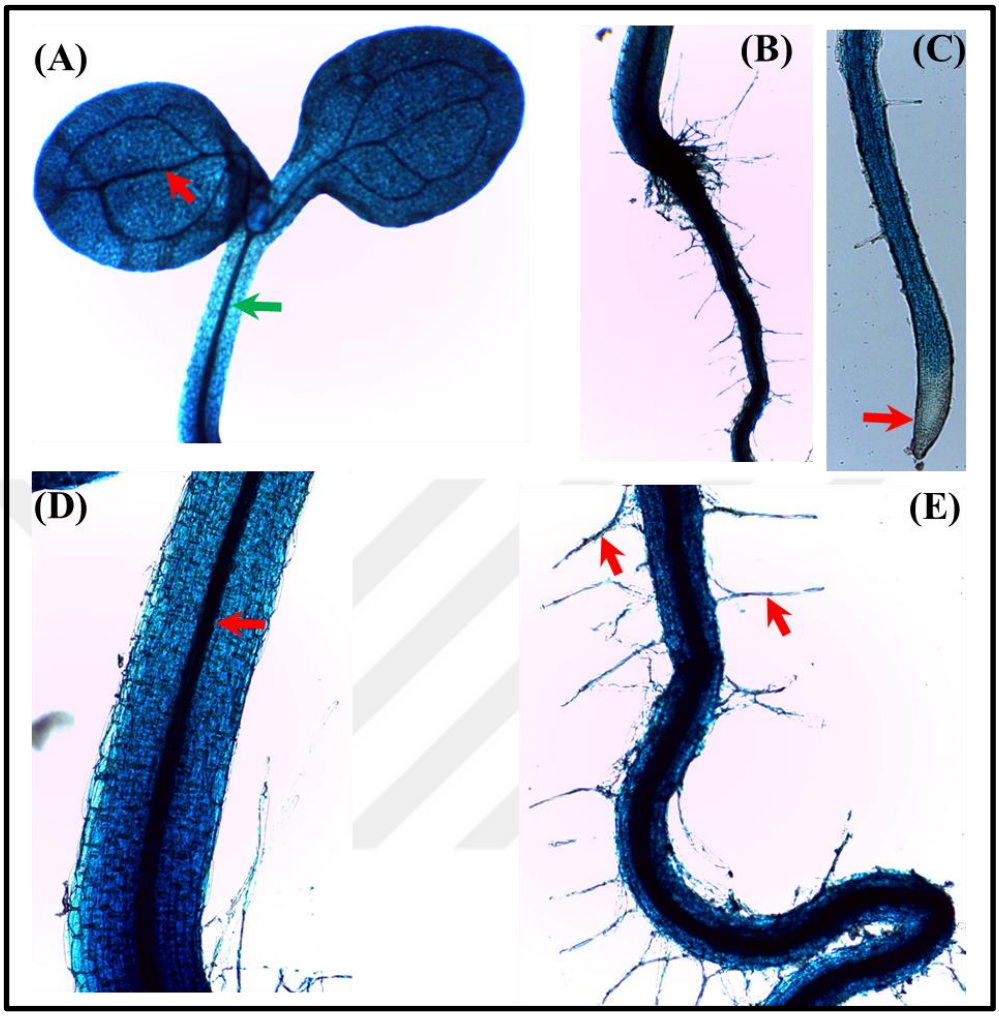

Figure 4.14. GUS histochemical staining of *5BPOR1promoter*::GUS in 5 days old Arabidopsis seedling

4.2.2.2 Ten days old seedlings

Ten days old seedling of *5βPOR1-promoter*::GUS was subjected to GUS histochemical analysis. Staining revealed that in 10 days old seedlings, strong expression activity of *P5βR1-promoter*::GUS was found in cotyledons, hypocotyl and root (Figure 4.15 A), close-up of cotyledons and hypocotyl (Figure 4.15 B), strong expression in vascular tissues indicated by red arrows (Figure 4.15 C), primary root (Figure 4.15 D), primary root close-up showing highest expression in vascular tissues (Figure 4.15 E), primary root with root tip (Fig. F) and close-up of root with meristematic zone (Figure 4.15 G). The promoter activity was found to be confined up to root elongation zone but not to meristematic zone where GUS expression was vanished. Further, the strongest promoter activity was found in vascular tissues (Figure 4.15 A-G) indicated by red arrow heads (Figure 4.15 C), although weak expression was also observed in cotyledons and hypocotyl tissues. These results show that, *5βPOR1promoter*::GUS has the highest expression activity at early seedling development stage in all tissues of cotyledon and hypocotyl, however, in root tissues only limited to vascular tissues but not in pericycle, endodermis, cortex and epidermis cells.

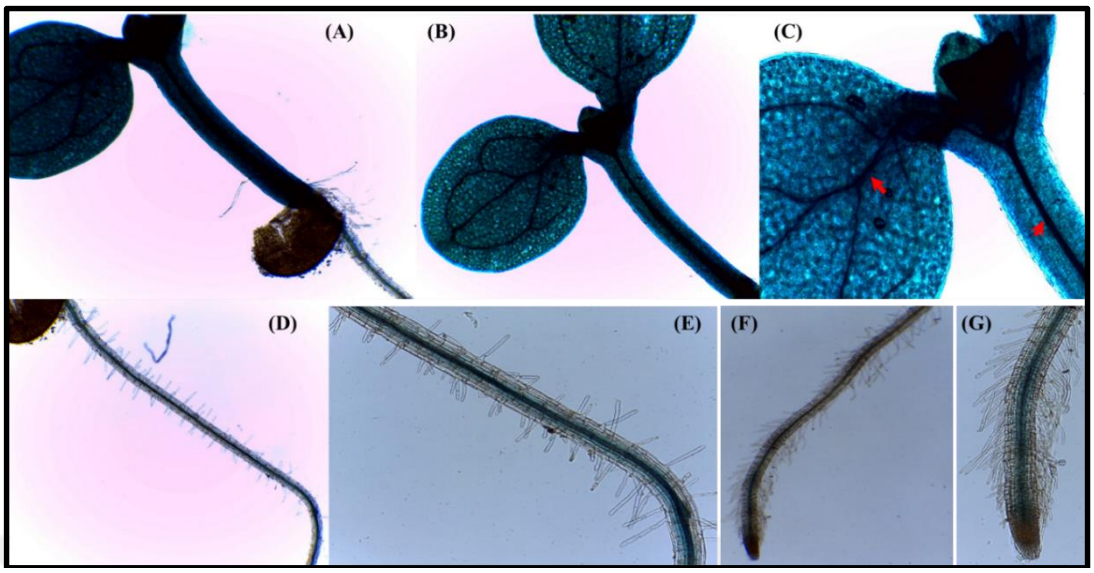

Figure 4.15. *5BPOR1promoter*::GUS activity in 10 days old seedling of Arabidopsis observed by GUS histochemical staining

4.2.2.3 Fifteen days old seedlings

Fifteen days old seedling of *5βPOR1promoter*::GUS was subjected to GUS histochemical analysis. Staining revealed that in 15 days old seedlings, in shoot tissues, strong expression activity of *P5βR1promoter*::GUS was found in cotyledons (but not in true emerging leaves) and hypocotyl (Figure 4.16 A), close-up of shoot tissues and hypocotyl shows strong expression in vascular tissues and weak expression in newly emerging leaves and mainly confined to hydathodes as indicated by yellow arrow (Figure 4.16 B), strong expression in hydathode indicated by yellow arrow in cotyledon (Figure 4.16 C and D). Further, strong expression was extended to periphery of hydathode indicated by red arc (Figure 4.16 D), In root tissues, weak expression was observed in primary root just below hypocotyl region (Figure 4.16 E) and primary root close-up showing highest expression in root hairs (Figure 4.16 F), slightly strong expression of promoter was found in vascular tissues and totally absent in emerging lateral root indicated by red circle (Figure 4.16 G) while close-up of lateral root shown in Figure 4.16 H clearly depicting absence of GUS activity in lateral root and root hair (indicated by black arrow). Promoter activity was totally absent in root differentiation zone, elongation zone, basal meristem and apical meristem tissues (Figure 4.16 I). These results show that, *5BPOR1promoter*::GUS has the highest expression activity at early seedling development stage in all tissues of cotyledon and hypocotyl, however, in root tissues only limited to vascular tissues but not in pericycle, endodermis, cortex and epidermis cells.

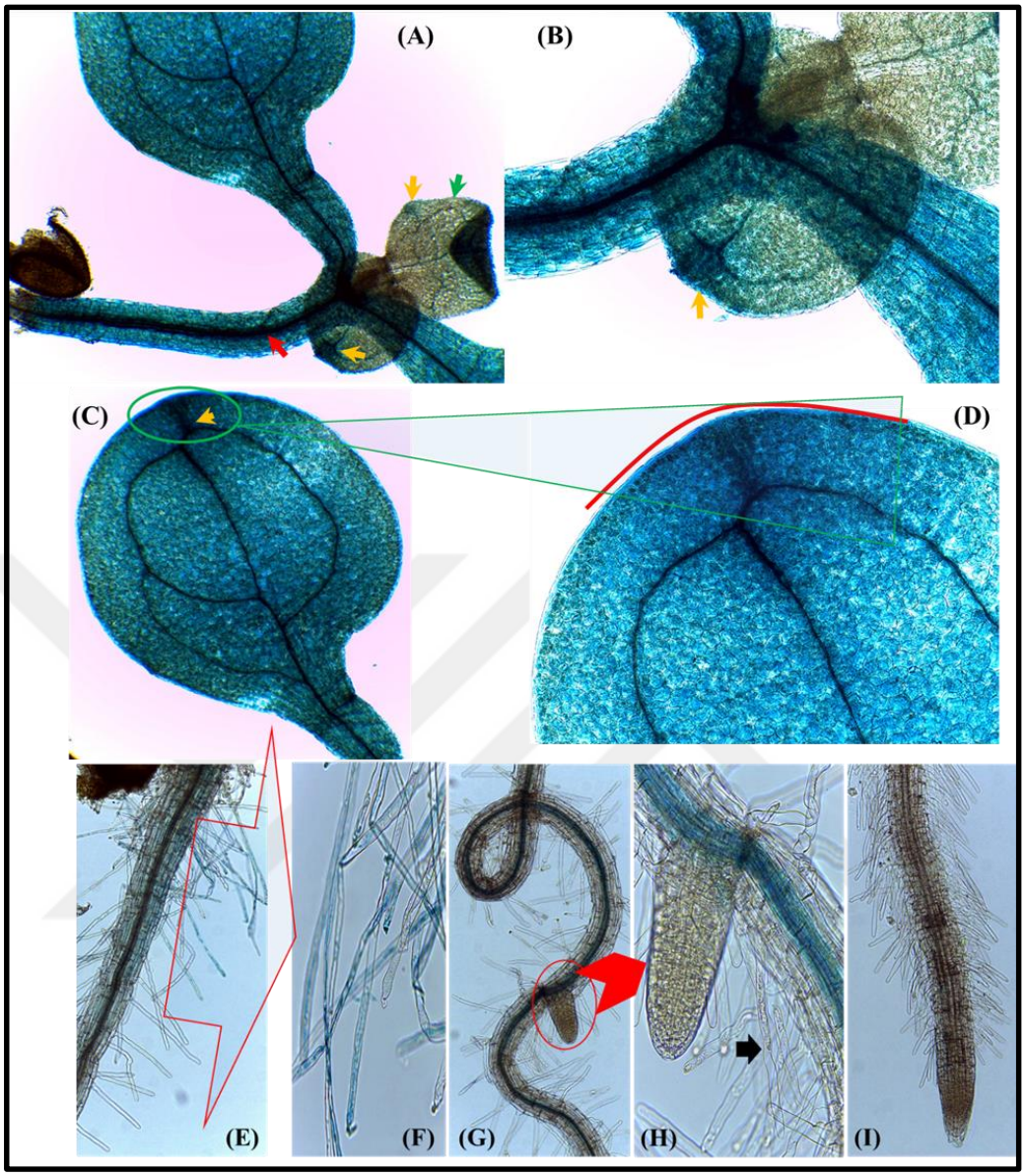

Figure 4.16. *5βPOR1promoter*::GUS activity in 15 days old seedling of Arabidopsis, observed by GUS histochemical staining. Upper frame of picture depicts promoter activity in shoot part while lower in root tissues.

5βPOR1promoter::GUS activity was the highest in 5 and 10 days old seedlings stages, however, expression intensity decreased slightly in 15 days old seedlings. Similar results reported for the promoter activity of *AtHSD1promoter*::GUS in Arabidopsis, the highest promoter activity was observed in germinating seedling and expression intensity decreased in newly merged leaves of plantlets (Baud et al. 2009). *AtHSD1* gene was reported to be related to oil bodies accumulation tissues and maturing seeds. *P5BR1* was reported to be a key gene in cardenolide biosynthesis pathway (Pérez Bermúdez et al. 2010). Further, *5βPOR1* activity at early seedling development suggests its dual role in growth and development stages as well as in cardenolide metabolism in mature leaf.

4.3 Cloning and analysis of the *5βPOR2promoter*

The promoter *5βPOR2* was cloned from genomic DNA library (DL3) digested with *Pvu* II restriction enzyme (Figure 4.17). The two bands were subcloned into cloning vector and the transformed bacterial colonies were selected by colony PCR (Figure 4.18). PCR product shown in Figure 4.18 as band number 1 gave precise sequence therefore the sequence (Figure 4.19) was considered for the downstream analysis.

The sequence (Figure 4.19) analysis of the *5βPOR2* promoter (558 bp) revealed the presence of *cis*-acting element involved in the abscisic acid responsiveness, light responsive module, light responsive element, elicitor-responsive element, MYB binding site, *cis*-acting element involved in defense and stress responsiveness, *cis*-acting regulatory element involved in the MeJA-responsiveness, *cis*-acting regulatory element required for endosperm expression and also core elements of promoter such as CAAT and TATA-box (Table 4.3). The *5βPOR2promoter* has the similar regulatory elements as the *5βPOR1promoter*. Such as, abscisic acid responsiveness, light responsive module, light responsive element, elicitor-responsive element, MYB binding site and *cis*-acting regulatory element required for endosperm expression as mentioned under the section Cloning and analysis of the *5βPOR1 promoter*. However, two *cis*-elements involved in defense and stress responsiveness (Klotz and Lagrimini 1996) and MeJA-responsiveness (Rouster et al. 1997) are found to be novel which are not present in *3β-HSDpromoter* and *5βPOR1 promoter*.

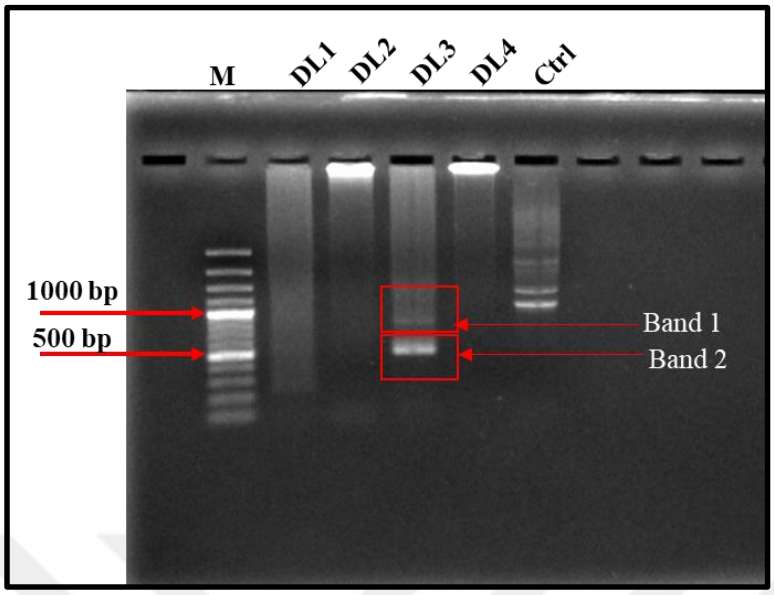

Figure 4.17. Cloning of the *5βPOR2 promoter* fragment. DL3 is gDNA library digested by *Pvu* II enzyme. Both bands were cut and gel purified for subcloning into cloning vector pCR2.1. *Dra* I (DL1), *EcoR* V (DL2), *Pvu* II (DL3) and *Stu* I (DL4).

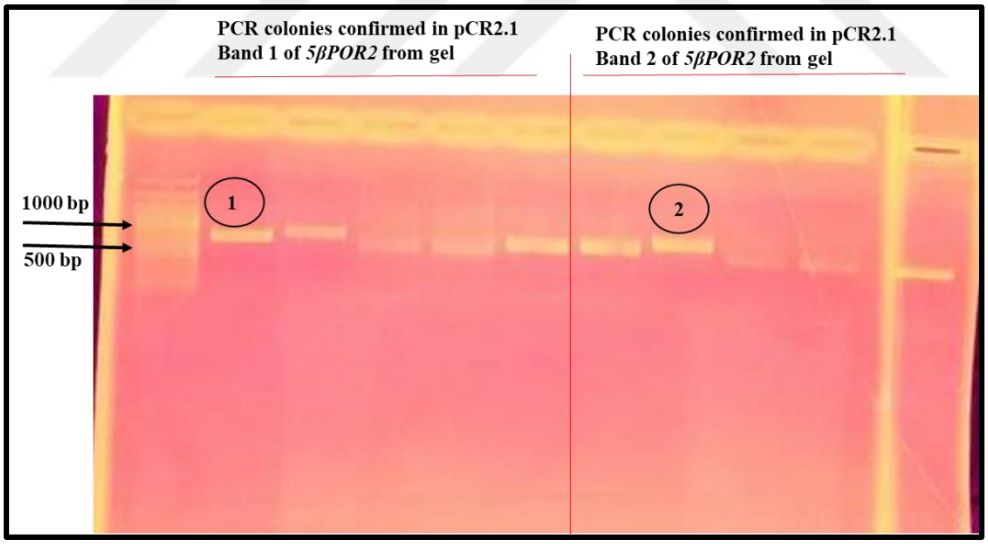

Figure 4.18. Selection of appropriate fragment of the *5βPOR2 promoter*. Clonoy 1 and clony 2 from gel band 1 and 2 respectively were propagated for plasmid isolation and *5βPOR2* sequencing. M13 forward and reverse primers were used

>Promoter *5βPOR2*

GGTCTGGCTGAAACTCTATCGAGCGGTCTGGCTGAAACTCTATCAAGTCCAAATGTCCAATAATAGTTGTTCCCCCTTTCTCCT
CCAGATCGAATCTCCAACTCCAACATTTATATTTTCTTCATCTTCTCCAGATCAGAAACAAATATTGAGAAAGCAACTTAATTAC
CTCAAAATCACGTAAATATATGCTAACCTAAGCAACGTTCAATTCATACAAGAAGACATGTTTATTTAACATGAAATGTCTTCAA
CTCAACCTGTAAATTCTACTTGATCTTTCCTGGGTCATAAAGAAAAGCACGAAAGCTAAAAGTAAGCAATACATCAAAATTTGT
AGGGGGAAAAAAAAAAACTACTACTAAATTGAAGCTGAGAAGATCATGATAACTTACTATTTTTGGTGCAAGGAACAAAATCT
GGAACCTTAAAGAGAGATTTCTGGCAAGCGCAGTGGAAGCGGTCTGGCTGAAACACTATCAATGCGGATGACGCGCCTGTA
GCCGCGCATAGCGCGCGGATGTGTGATTACGCGCACGTGAACGGCTTACACTGCCAG

Figure 4.19. Sequence (558 bp) of promoter fragment of the 5*βPOR2*

Table 4.3. Promoter fragment of *5βPOR2promoter*

Site Name	**Organism**	**Position**	**Matrix score**	**sequence**	**Fucntion**
3-AF1 binding site	*Solanum tuberosum*	429	10	AAGAG ATATTT	light responsive element
ABRE	*Arabidopsis thaliana*	177, 534	6 6	TACGGT C CACGTG	*cis*-acting element involved in the abscisic acid responsiveness
AE-box	Arabidopsis thaliana	137	8	AGAAAC AA	part of a module for light response
CAAT-box	*Brassica rapa* *Hordeum vulgare* *Glycine max* *Brassica rapa* *Arabidopsis thaliana* *Hordeum vulgare* *Brassica rapa*	49 320,147, 479, 57 363, 208 332 56 364 142	5 4 5 5 5 4 5	CAAAT CAAT CAAATT CAAAT CCAAT CAAT CAAAT	common *cis*-acting element in promoter and enhancer regions
CGTC-motif	*Hordeum vulgare*	488	5	CGTCA	elicitor-responsive element
G-box	*Antirrhinum majus* *Pisum sativum*	177 534	6 6	CACGTA CACGTG	*cis*-acting regulatory element involved in light responsiveness
G-box	*Daucus carota* *Arabidopsis thaliana*	177 534	6 6	TACGTG CACGTG	*cis*-acting regulatory element involved in light responsiveness
MRE	*Petroselinum crispum*	193	7	AACCTA A	MYB binding site involved in light

Table 4.3 (Continued)

					responsiveness
Skn-1_motif	*Oryza sativa*	287 487	5	GTCAT GTCAT	*cis*-acting regulatory element required for endosperm expression
TATA-box	*Glycine max* *Arabidopsis thaliana* *Lycopersicon esculentum* *Arabidopsis thaliana* *Daucus carota* *Brassica napus* *Daucus carota* *Arabidopsis thaliana*	60 185 111 310 109 259 184 331 110	5 4 5 6 9 6 9 5	TAATA TATA TTTTA TATAAA ccTATAAATT ATATAT ccTATAAATT TATAA	core promoter element around -30 of transcription start
TC-rich repeats	*Nicotiana tabacum*	114 123	10 9	ATTTTCTTCA ATTTTCTCCA	*cis*-acting element involved in defense and stress responsiveness
TGACG-motif	*Hordeum vulgare*	488	5	TGACG	*cis*-acting regulatory element involved in the MeJA-responsiveness
Unnamed__4	*Petroselinum hortense*	79 128 95 82 101	4	CTCC	

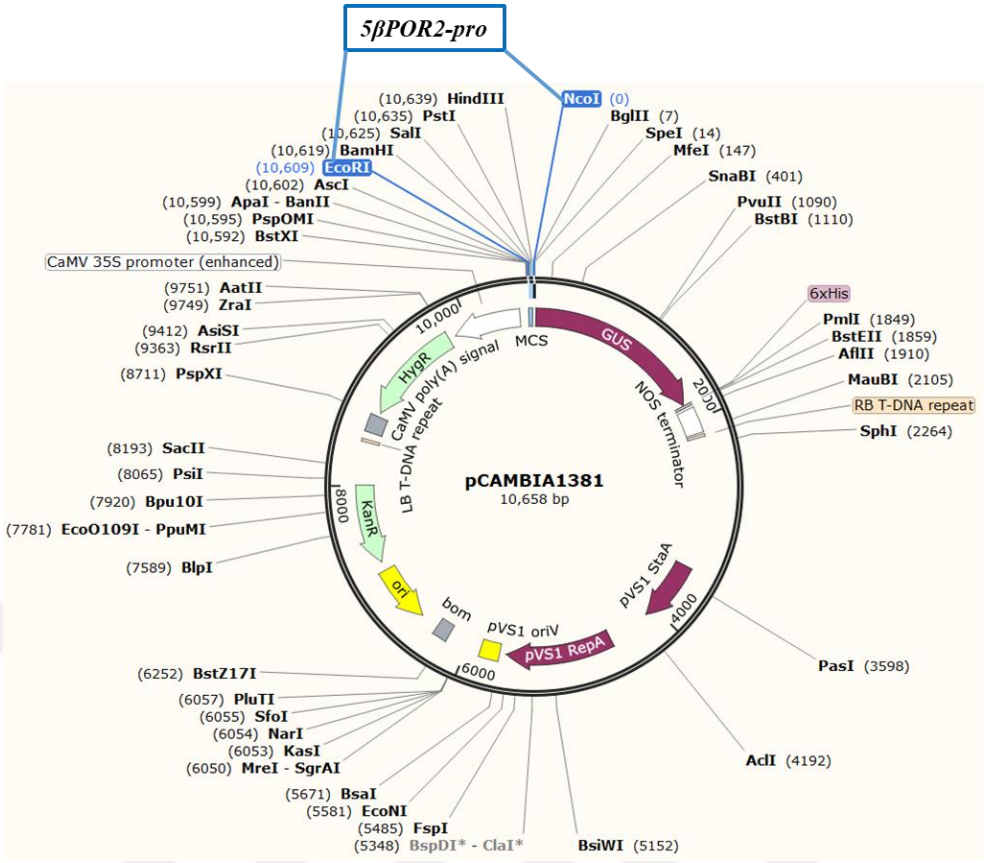

Figure 4.20. Insertion of 5*βPOR2promoter* into pCambia 1381 for the development of 5*βPOR2promoter*::GUS construct for Arabidopsis transformation.

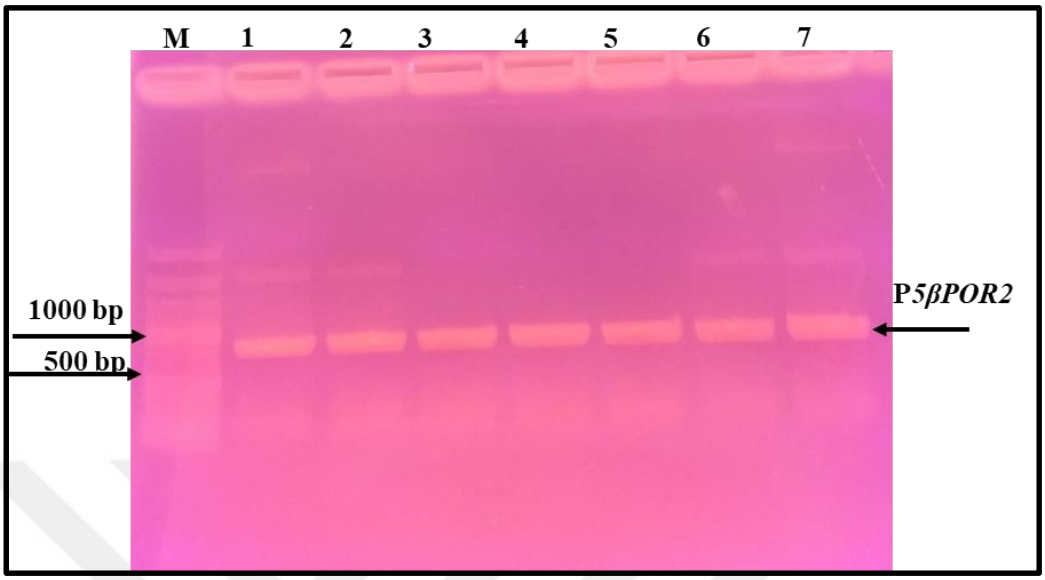

Figure 4.21. PCR verified promoter of *5βPOR2* in *Agrobacterium tumefaciens* colonies. *5βPOR2promoter* transformed into *Agrobacterium* and colonies 3, 4, 5 were selected for transformation experiments in future

4.3.1 Development of transgenic Arabidopsis plants carrying *5βPOR2promoter*::GUS

The promoter fragment was inserted into *Eco*RI and *Nco*I restriction sites of pCambia 1381 (Figure 4.20) and then transformed into *Agrobacterium tumefaciens*. The agrobacterium colonies were again verified by colony PCR (Figure 4.21) and transformed into Arabidopsis via floral dip transformation (Figure 4.21). The transgenic seedlings were selected on the ½MS agar media containing hygromycin during onset of true leaves (Figure 4.22 C and D). The T1 transgenic seedlings were transferred to sterile peat soil and covered with polythene sheet for acclimatization (Figure 4.22 E). One week later, polythene sheet was removed. The seedlings were watered with ½ MS media once a week. The seedlings were grown to seed maturity level and T2 seeds were harvested. The T2 seeds were grown aseptically on ½MS media (Figure 4.23) containing hygromycin and the seedlings (Figure 4.23) were selected at 5, 10 and 15 days' interval and promoter activity was observed.

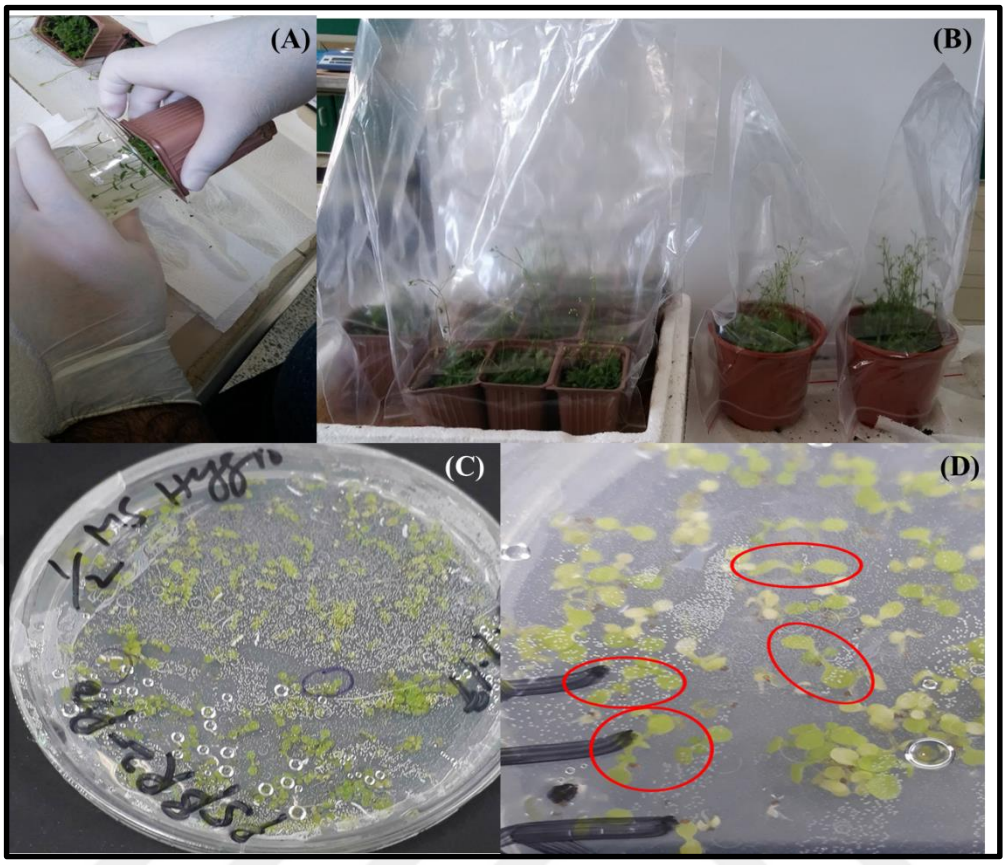

Figure 4.22. Development of transgenic Arabidopsis plants carrying promoter fragment of the *5βPOR2promoter::*GUS. (A) Floral dip transformation of Arabidopsis seedlings. (B) after transformation seedlings were covered with polythene bag to maintain high humidity over 24 h. (C) selection of transgenic Arabidopsis seedling on ½MS media containing 25 µg/mL hygromycin. (D) Second leaf emerged from transgenic seedlings and circled with red color.

(Figure 4.22 continued)

Figure 4.22. Development of transgenic Arabidopsis plants carrying promoter fragment of the *5βPOR2promoter::*GUS. (E) Transgenic Arabidopsis seedlings were transferred to sterile peat soil and covered with transparent polythene sheet for acclimatization. Red arrow shows seedlings in the pots.

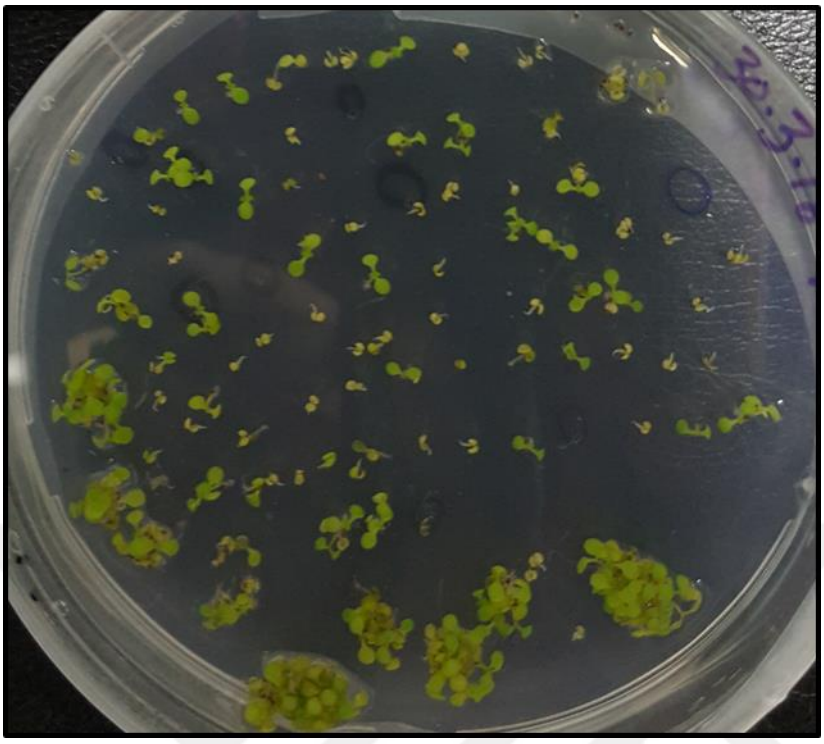

Figure 4.23. Transgenic Arabidopsis T2 seedlings of *5βPOR2promoter*::GUS used for GUS histochemical analysis. The seedlings used at 5, 10 and 15 days interval during seedling growth and development stages.

4.3.2 Activity of *5βPOR2promoter*::GUS during growth and development stages

4.3.2.1 Five days old seedlings

Five days old seedlings of *5βPOR2promoter*::GUS were subjected to GUS histochemical analysis. In shoot, staining revealed promoter activity in 5 days old seedling (Figure 4.24 A). Strong expression activity of *5βPOR2promoter*::GUS was limited to vascular tissue in cotyledons (Figure 4.24 B) indicated by red arrow, slight expression was observed in trichome cells over shoot apical meristem (indicated by dark blue arrow) while promoter activity was absent in shoot apical meristem (indicated by yellow arrow) and hypocotyl, indicated by green arrow (Figure 4.24 B). Close-up of cotyledon showing strong expression activity in vascular tissue (Figure 4.24 C). In root tissues, GUS histochemical staining revealed that promoter activity was totally absent in all tissues of root (lower frame, Figure 4.24 C-F).

These results show that, *5βPOR2promoter*::GUS has the highest expression activity in 5 days seedling of Arabidopsis was limited to vascular tissues in cotyledon, while absent in rest of all tissues.

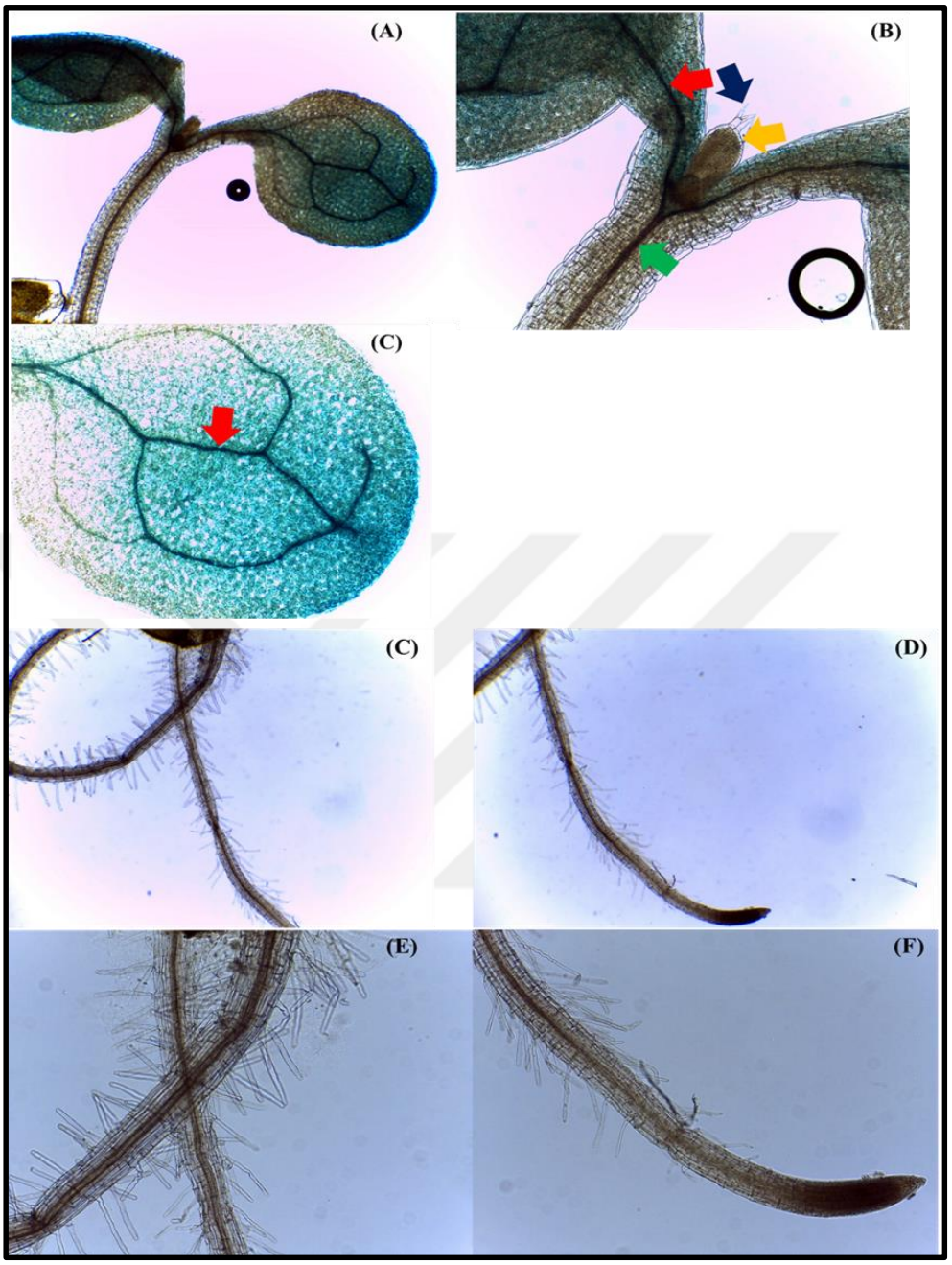

Figure 4.24. GUS histochemical staining of *5βPOR2promoter*::GUS in 5 days old Arabidopsis seedling.

4.3.2.2 Ten days old seedlings

In contrast to 5 days old seedling of *5βPOR2promoter*::GUS, 10 days old seedlings exhibited promoter activity extensively in tissues such as cotyledons, vascular tissues, hypocotyl and root tissues **(**Figure 4.25).

In shoot tissues, upper frame of figures, strong expression activity of *5βPOR2promoter*::GUS found in cotyledons (Figure 4.25 A), vascular tissues indicated by red arrow. Slight expression activity was found in shoot apical meristem indicated by big red arrow (Figure 4.25 B). Strong expression was also found in hypocotyl and root tissues (Figure 4.25 C).

In root tissues, lower frame of figures, promoter activity observed in hypocotyl and primary root tissues (Figure 4.25 D). Close-up of root tissues revealed that promoter activity confined to vascular tissues just below the adjacent area of hypocotyl (Figure 4.25 E). However, the promoter activity in the rest of root part was vanished and no activity was found in root tissues (Figure 4.25 F and G).

These results depict that, *5βPOR2promoter*::GUS has the highest expression activity in 10 days seedling of Arabidopsis was extended to cotyledons, vascular tissues in cotyledons and hypocotyl and to some extent in root tissues adjacent to hypocotyl. However, promoter activity was absent in the rest of root tissues.

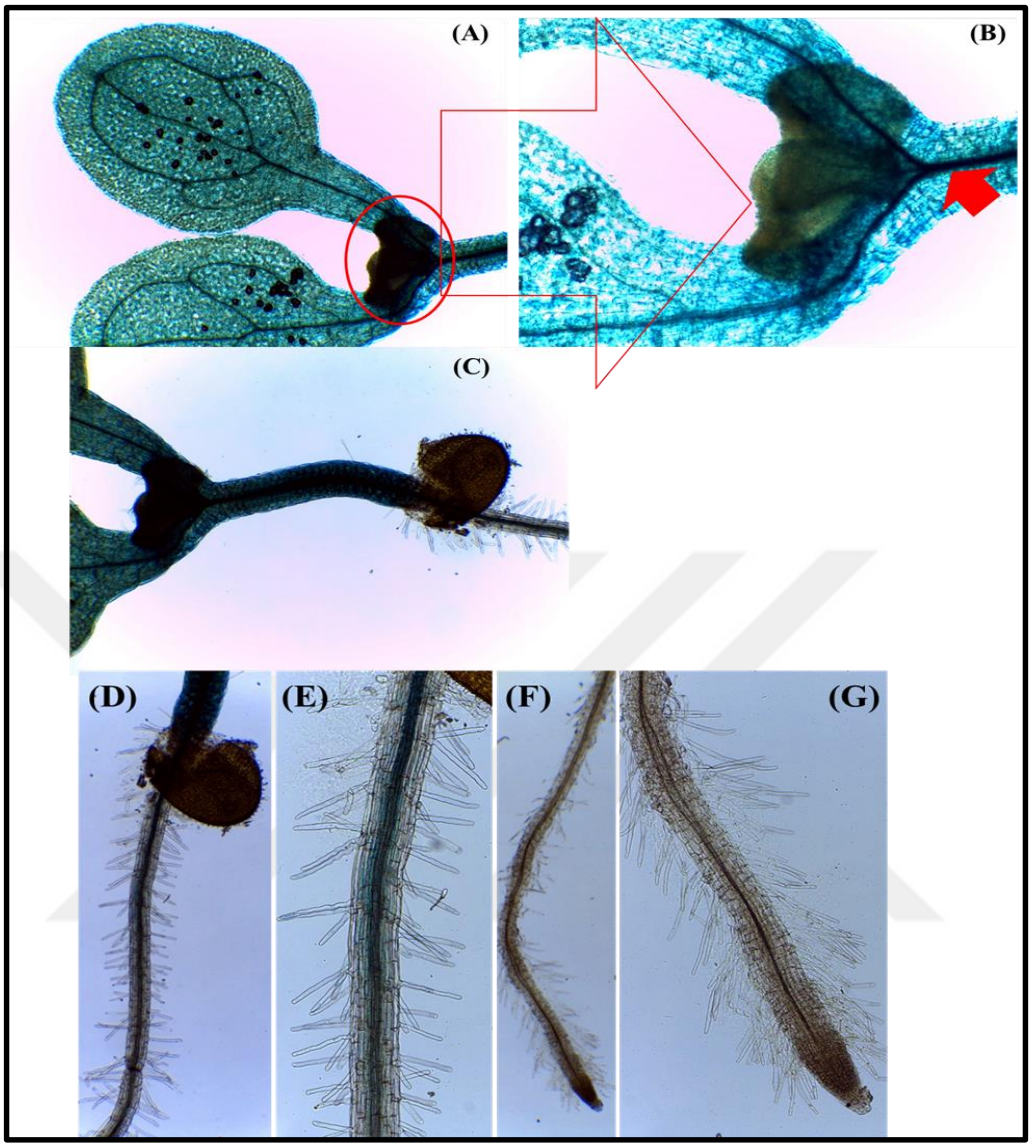

Figure 4.25. GUS histochemical staining of *5βPOR2promoter*::GUS in 10 days old Arabidopsis seedling.

4.3.2.3 Fifteen days old seedlings

GUS histochemical staining of 15 days old Arabidopsis seedlings showed that *5βPOR2promoter*::GUS activity was limited to hydathod in cotyledon tissue (Figure 4.26 A). Promoter activity was absent in shoot apical meristem (Figure 4.26 B), on the other hand, promoter expressed in hypocotyl region and strong activity was found in vascular tissues (Figure 4.26 C). Promoter activity was not observed in root tissues at all (Figure 4.26 D and E). *5βPOR2promoter*::GUS activity was diverse in 5, 10 and 15 days old seedlings. In 5 days old seedlings was found only in cotyledon and vascular tissues but absent in rest of the tissues including roots. However, in 10 days old seedlings, GUS activity was observed in cotyledons, vascular tissues, hypocotyl and root tissues adjacent to hypocotyl but absent in rest of root tissues. Again, in 15 days old seedlings, expression of the promoter was confined to hydathode in cotyledon, enhanced activity in vascular tissue of hypocotyl and absent in rest of tissues including SAM, root tissues.

These findings show that *5βPOR2promoter* expression oscillate with time during plant growth and development. The fashion of the promoter expression is somewhat similar to the *AtHSD1* which was also present in cotyledon or hypocotyl but absent in newly emerged leaf and root at all (Baud et al. 2009). In a previous report on *5βPOR2* (Pérez Bermúdez et al. 2010), the expression pattern of the gene was highly regulated by stresses such as wound, cold, heat, salt, ethylene hormone, methyl jasmonate and oxidative stress given by H_2O_2. The expression pattern in different tissues revealed that expression of *5βPOR2* was higher in leaf than root. These findings are in accordance with our promoter expression pattern during plant growth and development stages at 5, 10 and 15 days interval.

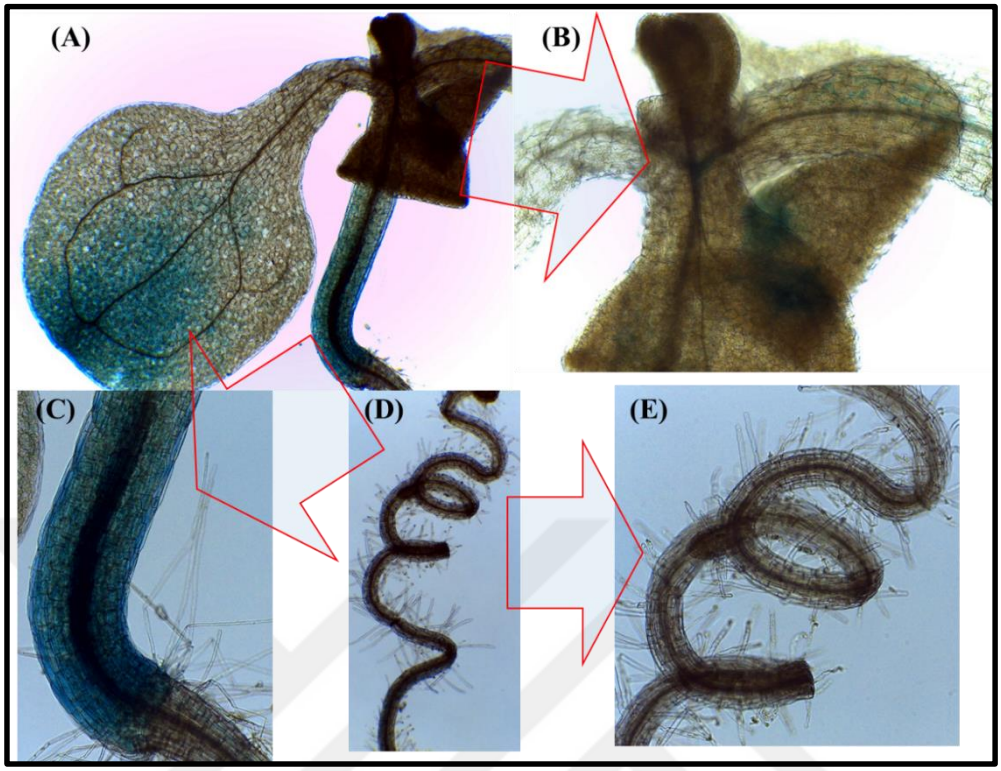

Figure 4.26. GUS histochemical staining of *5βPOR2promoter*::GUS in 15 days old Arabidopsis seedling.

5. CONCLUSIONS

The promoter fragments of key genes *3β-HSD, 5βPOR1* and *5βPOR2* in cardenolide biosynthesis pathway were isolated by genome walking DNA technique. The analysis of the promoter sequences revealed that these genes are highly regulated by light since light response elements were found in abundant in the promoter sequences. Therefore, for the proper regulation and expression of the genes light is important. Further, circadian clock, drought stress, stress and defense, elicitor response elements were found in the promoters of *3β-HSD, 5βPOR1* and *5βPOR2*. Heat stress element was found only in *5βPOR1*. Wound response element was found in the *3β-HSD* promoter, heat stress response element in the promoter of *5βPOR1,* and methyl jasmonate was in *5βPOR2.* Abscisic acid response element was found in both promoters of the *5βPOR1* and *5βPOR2*. Anaerobic induction elements were found in promoters of the *3β-HSD* and *5βPOR1*.

Functional characterization of the *3β-HSDpromoter*::GUS, *5βPOR1promoter*::GUS and *5βPOR2promoter*::GUS in Arabidopsis showed that the promoters have functional diversity. Expression activity of *3β-HSDpromoter* in Arabidopsis varies with age of the seedlings. Promoter activity enhanced with the age of seedlings and intensively expressed in 20 days old seedlings in shoot, root, trichomes, SAM, hypocotyl, lateral roots, root hair and roots. In the presence of mannitol and sucrose expression enhanced in seedlings in shoot and root while decreased in cotyledon in NaCl treatment compared to control.

5βPOR1promoter in 5 and 10 days old seedlings showed almost similar expression activity in cotyledon, SAM, hypocotyl and root but not in root apical meristem. However, in 15 days old seedlings, expression activity was weak in newly emerged leaves, intensive expression in vascular tissues of cotyledons, hypocotyl and root tissues. Moderate GUS activity was also found in root hairs. GUS activity was absent in lateral root and in root differentiation and elongation and apical meristem zones.

5βPOR2promoter in 5 days old seedlings showed expression activity limited to vascular tissues in cotyledons while absent in rest of the tissues of the seedlings. In 10 days old seedlings of Arabidopsis promoter activity was intensively found in cotyledons, vascular tissues of cotyledon, hypocotyl and adjacent root tissues.

Promoter activity was found with slight expression in SAM and totally absent in the rest of root tissues.

Taken together, these promoters (*3β-HSD*, *5βPOR1* and *5βPOR2*) are regulated by light and play roles in plant growth and development as well as in stress conditions. Further, the exact expression regulation of the genes *3β-HSD*, *5βPOR1* and *5βPOR2* is determined by promoter functional characterization.

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7. APPENDICES

APPENDICE A

Buffers

CTAB Buffer 1X, 200 mL

Chemical name	Amount	Concentration
CTAB	4 g	2%
1M Tris-Hcl	40 mL	200 mM
0.5 M EDTA	8 mL	20 mM
NaCl	16.36 g	1.4 M
PVP40	2 g	1%
The volume was filled to 200 mL with doubled distilled water and stored at +4°C		

TAE Buffer 50X, 1 L

Chemical name	Amount	Concentration
Tris	242 g	2 M
Acetic acid	7.1 ml	0.7 %
0.5 M EDTA	100 ml	0.05 M
The volume was filled up to 1 L with double distilled water and then autoclaved.		
TAE Buffer 1X, 1 L The buffer TAE 50X was diluted 1:50 in double distilled water.		

TE buffer

Chemical name	Amount	Concentration
Tris-HCl (1M, pH 8.0)	1 mL	10 mM
EDTA (0.5 M, pH 8.0)	200 µL	1 mM
Ultra pure H_2O	98.8 mL	
Store at room temperature.		

APPENDICE B

Stock Solutions

EDTA 0.5 M, pH 8.0, 1000 ml	168.1 g EDTA 22.5 g NaOH dissolved in 850 ml distilled water and autoclaved.
Tris-HCl 1 M, 1 L	Tris (121.1 g) was dissolved in 1000 ml distilled water and pH was adjusted with conc. HCl
MS-media, pH 5.8, solid, 1 L	MS media salt (4.4 g) including vitamins 30 g Sucrose 8 g Agar Components were dissolved in 1 L distilled water and pH adjusted to 5.8 and sterilized by autoclaving.
NaCl 1 M, 1 L	58.44 g NaCl were dissolved in 1 L distilled water and autoclaved.
NaCl 5 M, 500 ml	146.4 g NaCl were dissolved in 1 L distilled water and autoclaved.
50 mg/mL Kanamycin Stock Solution	Weighed 1 g of Kanamycin Added 20 ml of sterile H2O. filter sterilized by 0.22 µm syringe filter by drawing through 5-10 ml of sterile H2O and discard water.
50 mg/mL Hygromycin Stock Solution	Weighed 1 g of Hygromycin B and added 20 ml of sterile H2O. after dissolving sterilized through the 0.22 µm syringe filter and store at -20°C.

APPENDICE C

Media

LB-Medium, pH 7.0, liquid, 1 L	10 g Bacto Tryptone 5 g Yeast extract 10 g NaCl Mixed and dissolved in 1 L distilled water, pH adjusted to 7.0 and sterilized by autoclaving.
LB-Medium, pH 7.0, solid, 1 L	10 g Bacto Tryptone 5 g Yeast extract 10 g NaCl 10 g Bacto-Agar Mixed and dissolved in 1 L distilled water, pH adjusted to 7.0 and sterilized by autoclaving.
MS-medium, pH 5.8, solid, 1 L	4.4 g MS-Salt including vitamins 10 g Sucrose 8 g Agar powder Mixed and dissolved in 1 L distilled water, pH adjusted to 5.8 and sterilized by autoclaving.
SOC media	0.5% (w/v) Yeast extract 2% (w/v) Tryptone 2.5 mM KCl 10 mM NaCl 20 mM Glucose 10 mM $MgCl_2$ The solution containing the first four components, sterilize at 121 °C, and then added sterile $MgCl_2$ and glucose.

APPENDICE D

ENZYMES AND OTHER CHEMICALS

Chemical name	**Company name**
*Eco*RI	New England BioLab
*Nco*I	New England BioLab
T4 DNA ligase	New England BioLab
6X Loading dye	Thermo Scientific
X-Gluc	Thermo Fisher Scientific
Silwet L-77	lehle seeds
GenomeWalker™ Universal Kit	Clontech, CA, USA)
Plasmid isolation kit	Thermo Scientific
TA cloning kit	Thermo Scientific
NucleoSpin® Gel and PCR Clean-up kit	(Macherey-Nagel, Germany).

APPENDICE E

EQUIPMENTS USED IN THIS STUDY

Equipment name	**Company name**
-80°C deep freezer	Thermo Scientific, USA
-20°C deep freezer	Vestel
28°C and 37°C incubators	Nuve FN 500
28°C and 37°C shaking incubator	Gerhardt
+4°C refrigerator	Vestel
Autoclave	Nuve
Centrifuge	Hettich Micro 120
Gel electrophoresis system	Thermo Scientific
Power supply EC 250-90	Thermo Scientific
Imaging system	UVP Photo Doc-It™
Micropipettes	Finnipipette
pH meter	HANNA HI 221
Spectrophotometer	HITACHI U-1900
Water purification system	Merck Millipore system
vortex	Yellow line TTS2
T100™ Thermal Cycler	BIO-RAD, USA
Microscope	Leica DM1000 LED

8. CURRICULUM VITAE

Name SURNAME **:** Noreen ASLAM

Place and Date of Birth **:** Khanewal, Pakistan 04/01/1990

Universities

Bachelor's Degree **:** Botany, Bahauddin Zakariya University, Multan, Pakistan

MSc Degree (varsa) **:** Plant Biotechnology, Abant İzzet Baysal University

e-mail **:** noreensamee206@gmail.com

List of Publications **:**

1) Muhammad Sameeullah, **Noreen Aslam,** Buhara Yucesan, Ekrem Gurel (2015) Dynamic Implementations of Plant Tissue Culture Techniques for Secondary Metabolite Production and Breeding Strategies. International Conference on Agronomy and Horticulture (ICAH 2015), Shanghai, China. Oral presentation.
2) Muhammad Sameeullah, Tijen Demiral, **Noreen Aslam,** Faheem Shehzad Baloch, Ekrem Gurel (2016) *In Silico* Functional Analyses of SWEETs Reveal Cues for Their Role in AMF Symbiosis. Plant, Soil and Microbes: Volume 2: Mechanisms and Molecular Interactions Editors Hakeem Rehman Khalid, Akhtar Sayeed Mohd Springer International Publishing. Pages 45-58.
3) Muhammad Sameeullah, Faheem Ahmed Khan, Goksel Özer, **Noreen Aslam,** Ekrem Gurel, Muhammad Tahir Waheed, Turan Karadeniz. (2017) CRISPR/Cas9-Mediated Immunity in Plants Against Pathogens. Current Issues in Molecular Biology. 2017 Sep 7;26:55-64. doi: 10.21775/cimb.026.055.