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Characterisation of actin depolymerising factor promoter (pTZPaADF) from *Plectranthus amboinicus* Lour (Spreng) using *Arabidopsis thaliana* (L.) Heynh as an internal control

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ABSTRACT

Plant genetic engineering heavily relies on transgenic technology. For the development of an effective vector system for genetic transformation application well-characterized promoter is crucial. Currently, available promoters are either viral or bacterial origin and hence it is necessary to isolate and analyze additional constitutive promoters of non-viral origin. Plant actin depolymerizing factors (ADFs) have diverse metabolic characteristics however, the majority of biological functions are yet unknown. In this study, we attempted to identify the promoter region of ADF from *Plectranthus amboinicus* using *Arabidopsis thaliana* as an internal control by thermal asymmetric interlaced polymerase chain reaction (TAIL PCR). Three heterologous gene-specific primers were designed to isolate the promoter region and the sequences were then examined *in silico* to check for the presence of minimal promoter elements. Our findings suggested that the TAIL PCR using specific primers is an efficient method for plant promoter isolation. The clone (pTZPaADF) carrying the putative promoter sequence was verified by comparing it with the results obtained for *A. thaliana*. Alignment of the putative promoter sequences with *Arabidopsis* revealed the presence of numerous conserved and shared RNA polymerase binding sites. *In silico* analysis revealed that there were several putative motifs including the TATA and the CAAT boxes. In addition, many other putative *cis*-acting regulatory elements that may regulate gene expression were also found. Based on the analysis the isolated putative promoter, pTZPaADF, could be employed in driving transgene expression.

1. INTRODUCTION

The multi-stage process of eukaryotic gene expression is regulated at the transcriptional, post-transcriptional, translational, and post-translational stages. Numerous *cis*-elements and *trans*-factors control transcription, the first stage of gene expression, and one of the most significant intracellular processes controlling gene expression. The direction, efficacy, and type of RNA polymerase that binds to the eukaryotic promoter and begins transcription are all controlled by this crucial sequence or center of transcription regulation [1]. A eukaryotic promoter is a DNA sequence normally located upstream of the transcribed region [Figure 1]. It contains TATA box and serves to determine the start site of transcription [2]. Studies of promoters that primarily control gene expression at the transcriptional level are essential for strengthening our fundamental understanding of gene regulation and will increase the variety of promoters that are available for use in plant biotechnology. The most important actin-binding proteins that significantly influence the alteration of actin cytoskeletal architecture are plant actin-depolymerizing factors and were found to be conserved in eukaryotes [3]. Actin depolymerizing factor has distinct biochemical and metabolic properties despite co-evolving with actin. The majority of plants actin-depolymerizing factors (ADFs) still lack a comprehensive understanding of how they work. ADF has been studied in many plants, including lilies [4], moss [5], rice [6], and Zea mays [7], Arabidopsis [8]. ADFs play major roles in the growth and development of plants; involved in the biotic and abiotic stress of plants [9,10]. Reports also suggest that ADFs are upregulated at low temperature [11] Though co-evolved with actin, the biochemical and metabolic characteristics of the ADF are different. With recent advancements in genomics, numerous technological processes have emerged enhancing a variety of plant molecular research. Although there has been a significant increase in plant genetic information, which are limited to model plants and few agronomically important plants, not much information is available for the medicinally important plants.

The present study aimed to isolate the promoter region of the ADF from *Plectranthus amboinicus* using *Arabidopsis thaliana* as an

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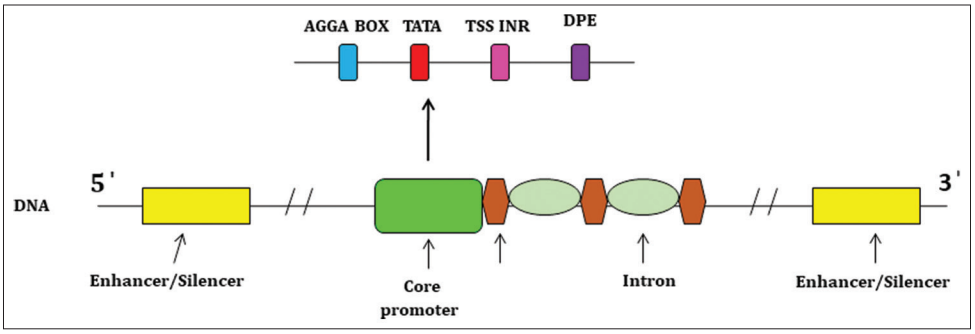

Figure 1: Structure of eukaryote promoter.

internal control by Thermal asymmetric interlaced polymerase chain reaction (TAIL PCR) and validate the generated sequences for basal promoter elements using computational analysis.

P. amboinicus is a perennial, tender, fleshy plant in the *Lamiaceae* family with an oregano-like flavor and aroma [Figure 2]. It is an ancient Ayurvedic plant that is a member of the mint and lavender family and grows in the mountains of Asia. The plant originated in South and East Africa, but it is now widely grown and naturalized in the tropics of both the Old and New Worlds. It is also known as Indian Borage, Mexican thyme, Mexican mint, Spanish thyme, *Cuban oregano*, or *Oregano brujo* (Puerto Rico). syn. Lour's *Coleus amboinicus* Benth is a *Coleus aromaticus*. Due to the anticipated rise in demand for its medicinal compounds, which are widely used to treat glaucoma, cardiac issues, and certain types of cancer, the crop has the tremendous potential [12].

2. MATERIALS AND METHODS

2.1. Plant Material and Growth Conditions

Wild-type *A. thaliana* (Col-0) seeds were provided by the NIPGR, New Delhi, India were used for initiation in pots containing a soil mix of vermiculite, river sand, and activated charcoal in the ratio 1:3:1. Seeds were then surface sterilized using hypochlorite (1%) and then used for initiation on soil mix. The pots were placed in the dark for 3–4 days to allow seed germination. The pots were then transferred to the light racks, the plastic wraps were removed and seedlings were allowed to grow. The plantlets were grown at 21°C with 16 h light and 8 h of darkness cycle in a light rack. *P. amboinicus* was collected from the herbal garden at Sri Ramachandra Institute of Higher Education and Research Institute and was authenticated by Prof. P Jayaraman at Plant Anatomic Research Centre, Tambaram, Chennai.

2.2. Isolation of Genomic DNA

Young leaves of the plant *P. amboinicus* were harvested and total genomic DNA was isolated following a modified procedure in the Winter's protocol [13]. All surface particles of the leaf samples were removed by washing them with distilled water. A 0.5 g of leaf samples were weighed powdered with liquid nitrogen and transferred to a clean microfuge tube. To this was added 500 µL of extraction buffer along with 33 µL of 20% SDS. The tube was gently swirled completely inverted to mix the sample and incubated at 65°C for 10 min. After incubation, 160 µL of potassium acetate was added and mixed well. The reaction mixtures were centrifuged at 10,000 rpm for 10 min and the aqueous phase was carefully transferred to 2 mL microcentrifuge tubes. 500 µL of chloroform was added to each tube and gently inverted until an emulsion formed. Samples were centrifuged at 10,000 rpm for 5 min and the aqueous phase was transferred into microfuge tubes. The

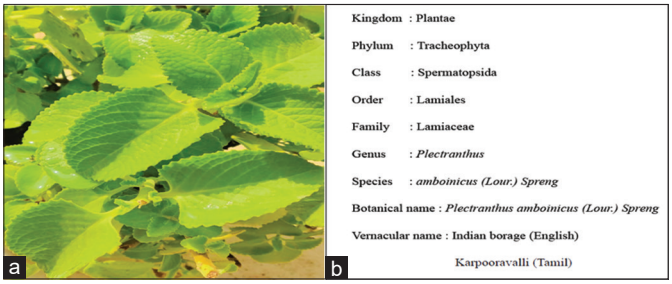

Figure 2: (a) *Plectranthus amboinicus* (Lour) Spreng. (b) Scientific classification of *P. amboinicus* (Lour) Spreng.

Table 1: Primers used for TAIL polymerase chain reaction.

Primer	Primer sequence
ADF I	5′ ATAGTGAATCCCTTCCAACACTC 3′
ADF II	5′ ATATGGGCATATGGCGTTTCTAA 3′
ADF III	5′ TACGTGACTTCTCTTCGATCTTG 3′
AD 1	5′ NTCGA (G/C) T (A/T) T (G/C) G (A/T) GTT 3′
AD 2	5′ NGTCGA (G/C)(A/T) GANA (A/T) GAA 3′
AD 3	5′ (A/T) GTGNAG (A/T) ANCANAGA 3′
AD 4	5′ TG (A/T) GNAG (G/C) ANCA (G/C) AGA 3′

TAIL: Thermal asymmetric interlaced, ADF: Actin depolymerising factor

chloroform wash was repeated, the aqueous phase was transferred to a new tube and 500 µL of cold isopropanol was added. The tubes were mixed well and incubated at RT for 10 min followed by centrifugation at 10,000 rpm for 5 min. The supernatant was discarded and the DNA pellet was washed twice with 500 µL of cold 70% ethanol. The DNA pellet was then air-dried and suspended in Milli Q water. Absorbances were recorded at 260 nm and 280 nm, to determine DNA quality and quantity. The DNA was then resuspended in MilliQ water and adjusted to a final concentration of 30 ng/µL.

2.3. Isolation of 5′ Region Using TAIL-PCR

TAIL-PCR was used to isolate the 5′ regions of ADF from *Plectranthus* and *Arabidopsis* plants. For this purpose, we selected three gene-specific primers from the published complete cds, NC_003071 REGION: 7241000.7246000 (*A. thaliana* chromosome 2, Actin depolymerizing factor 5) using the NCBI pick primer software. Four arbitrary degenerates (AD) primers AD1, AD2, AD3, and AD4 designed according to Liu and Whittier were used [14] [Table 1].

The TAIL PCR consisted of three consequential reactions primary, secondary, and tertiary reactions and utilized three gene-specific

primers in a nested way together with a short AD primer having a lower Tm to allow thermal control over the relative amplification of specific and non-specific products [Figure 3]. The primary reaction was performed using ADF I and one of each AD primer. Secondary and tertiary reactions were performed with ADF II and ADF III respectively using the product of the previous reaction as a template and using the same AD primers used in the primary TAIL PCR step. A 20 µL reaction mixture was prepared for primary TAIL PCR with 30 ng/ µl of genomic DNA, 0.2 µM gene-specific primer (ADF I), 3.0 µM of AD primer, and PCR Amplicon *Taq* DNA polymerase 2X Master mix. Secondary TAIL PCR was carried out with 20 dilutions of the primary PCR product containing ADF II and tertiary PCR was performed on 20 dilutions of the secondary PCR product. Cycle conditions are shown in [Table 2]. Final PCR products were separated by electrophoresis on a 1.5% agarose gel. The PCR-amplified DNA fragments were sequenced after cloning into pTZ57R/T using Thermo Scientific InsTAclone PCR Cloning Kit #K1213 [15].

2.4. Bioinformatic Analysis of the Promoter Sequence

The homology of the obtained sequences was analyzed using the Basic Local Alignment Search Tool (BLAST) (BLAST [nih.gov], Ensembl plant database(http:/plants.emsembl.org/Multi/Tools/Blast) [16]. The *cis*-regulatory elements of the sequences were analyzed using New PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/) [17], a database of plant *cis*-acting regulatory DNA elements PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [18]. The transcription start site (TSS) of the sequences was predicted

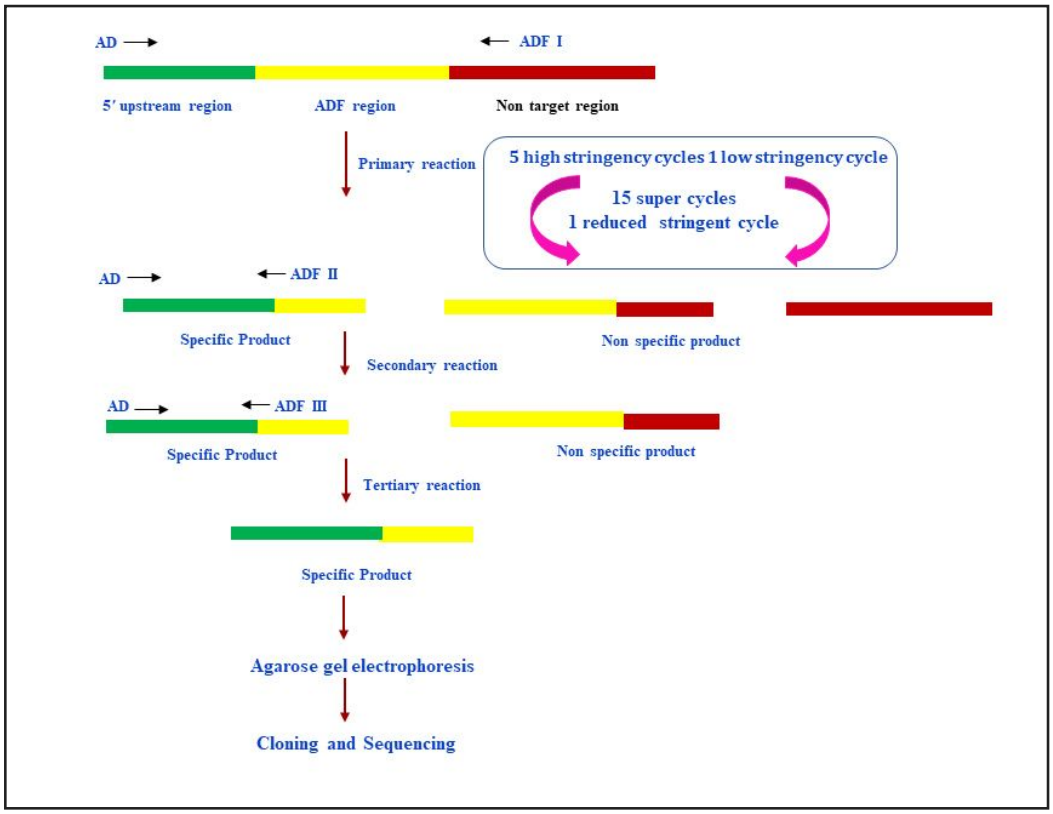

Figure 3: Schematic representation of thermal asymmetric interlaced polymerase chain reaction.

Table 2: Reaction condition for TAIL PCR.

Reaction	Program No	Number of cycles	Cycle parameters
Primary PCR	1	1	93°C, 1 min
	2	5	94°C 30 s; 60° C 1 min 72°C 2.30 sec
	3	1	94°C 30 s; 25°C, 3 min ramping to 72°C over 3 min
	4	15	94°C 30 s; 58.9°C 1 min; 72°C, 2 min 30 s; 94°C 30 s; 46.6°C 1 min; 72°C 2 min 30 s;
	5	1	72°C, 5 min
Secondary PCR	6	12	94°C, 10 s; 57.1°C,1 min 72°C, 2 min 30 s: 94°C ,10 s; 64°C, 1 min; 72°C, 2.5 min; 94° C, 10 s; 29°C, 1 min; 72°C, 2 min 30 s
	5	1	72°C, 5 min
Tertiary PCR	7	20	94°C, 15 s; 58.9°C, 1 min , 29°C, 30 s; 72°C, 2 min
	5	1	72°C, 5 min

TAIL: Thermal asymmetric interlaced, PCR: Polymerase chain reaction

using TSSPlant (http://www.softberry.com>berry>topic=tssp) [19] ENDMEMO is used to predict AT percentage (EndMemo - Online Converters, Calculators and Tutorials).

3. RESULTS AND DISCUSSION

3.1. Isolation of Genomic DNA

We extracted total genomic DNA from young leaves of *P. amboinicus* and *A. thaliana* using a modified Winter's protocol. The quality and purity of the isolated DNA were analyzed using a 1% agarose gel and quantified using a Nanodrop spectrophotometer. Intact genomic DNA was observed on 1% agarose gel and the purity at 260/280 was found to be 2.04 for *Plectranthus* and 2.08 for *Arabidopsis,* respectively. The isolated genomic DNA was used directly for TAIL PCR method for promoter isolation.

3.2. Isolation of Promoter Sequence Using TAIL PCR

TAIL PCR was performed using a combination of three nested gene-specific primers from ADFs along with the combination of four different AD primers that allow chromosomal walking beyond the 5' region. Three rounds of PCR primary, secondary and tertiary reactions were performed using the results of the previous PCRs as a template for the next reaction. All the products primary, secondary, and tertiary were examined, on 1.5% agarose gel electrophoresis using 100 bp DNA ladder as a standard. Progressive changes in PCR product sizes served as evidence of successful genome walking experiments [Figure 4a and b]. PCR products' sizes, ranging from 200 bp to 1.5 Kb, indicated that the amplification process of TAIL PCR was successful.

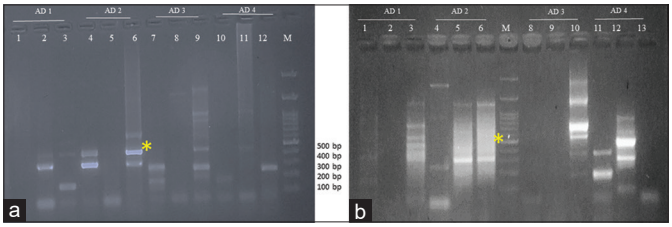

Figure 4: Agarose gel electrophoresis of thermal asymmetric interlaced polymerase chain reaction products of (a) *Plectranthus amboinicus* and (b) *Arabidopsis thaliana* amplified with different combinations of AD primers with respect to gene specific primers for primary secondary and tertiary reactions Product size of approximately 1 Kbp was observed with AD 2 primer indicated with an asterisk was cloned and sequenced. M indicates the Gene 1 Kb DNA marker.

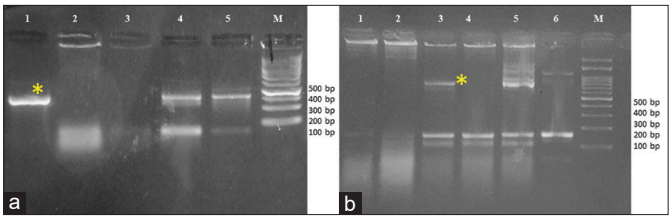

Figure 5: Confirmation of transformed colonies by colony polymerase chain reaction analysis in (a) *Plectranthus* (b) *Arabidopsis* using M13 forward and reverse primers.

3.3. Isolation of Promoter Sequence Using TAIL PCR

TAIL PCR was initially developed to screen YAC and P1 libraries in 1995 [20]. It has since been effectively applied to recover genomic sequences flanking the 5′ upstream region of DNA. The advantages of the method are it is simple, specific, efficient sensitive, and highly reliable over other PCR-based genome walking techniques. TAIL PCR was performed using a combination of three nested gene-specific primers from ADFs along with the combination of four different AD primers that allow chromosomal walking beyond the 5' region. To ensure the specificity and the efficiency of the primers and the lack of genome information on *P. amboinicus*, *A. thaliana* col0 wild type was used as an internal control for the study. Three rounds of PCR primary, secondary and tertiary reactions were performed using the results of the previous PCRs as a template for the next reaction. In the primary reaction using gene-specific primer ADF I, one low stringency cycle had been run to generate one or more annealing sites in the targeted sequence for the AD primer. The subsequent interspersion of two high-stringency PCR cycles with one reduced-stringency PCR cycle amplifies particular products over non-specific ones. It has a low yield of non-targeted products, a medium yield of targeted goods, and a high production of untargeted products. The gene-specific primers ADF II and ADF III used in the secondary and tertiary reactions produced no amplification of non-targeted products, extremely high yields of particular products, and very low yields of non-specific products. All the products primary, secondary, and tertiary were examined, on 1.5% agarose gel electrophoresis using 100 bp DNA ladder as a standard. Progressive changes in PCR product sizes served as evidence of successful genome walking experiments [Figure 4a and b]. PCR products' sizes, ranging from 200 bp to 1.5 Kb, indicated that the amplification process of TAIL PCR was successful. Among all the tested AD primers the products obtained from AD2 were discrete and distinct.

3.4. Cloning and Colony PCR Analysis of the Transformants

The PCR products obtained from the tertiary reactions were ligated into an appropriate cloning vector (pTZ57R/T) using the TA cloning

>p2PAAD2AEAD_m13Forward_9871-2_P1549,Trimmed Sequence(568bp)
TATATGGGCATATGGCGTTTCTAAGCAGAATTTCTTGATGAAATCCTTTGTATACTTTTTTTGACTGATGAAATTCCTTCAAATGACTAGTTG
ACTTGAAGTCCTAAGAAATATGTCAGTTTGCCCATCATACTCATTTGAAAATTTTGTTGCATCAATTGGGCAAATTTCTCGCATAGTGACTGA
TTCTTGGATCCAAGATTATGTCATCCACATAAACTAGCACAAGAAGAATATTGTCATTATCCTTTATGAGAAAGAGAGTCCAGTCGATCTTTC
CCTTTTTAAATCTATTCTTCAACGAAAATTGAGACAACGTTTCCTACCAAGCTCTGAGAGCTTGTTTTAATCCATAGAGAACTTTCCTTGGTC
TGTAGACTTTATCTGCTTCTTCAAAACCAAGAGGTTGTTCTACATATACTTCTTCATTCAGCTCTCCATTTAGAAACGCCATATGCCCATATT
ATCGGATCCCGGGCCCGTCGTTTGCAGAGGCCTGCATGCAAGCTTTC

a

>ADF.9_m13Forward_10433-3_P1605,Trimmed Sequence(766 bp)
CTAGATTATATGGGCATATGGCGTTTCTAACAAAAAAGTTTACAAGGGTGAGTTTGTAATAAGAGTAAATAGTGATCGCATTTGTATAGGCATAACTT
TTTATATAAAGACCTCAAAGTCTTACCATTTGAACAGAAAAAAGCTCTCCACGCCTAAAAAAAAAGAAAAAAGAGACAGAAGAAGAAAAAGATAAAGA
AGAATCAAACTTTGTAACCCTTTAGCAGCAATGGCGATGGCTTTCAAGATGGTAAATTCAAATCTGTTTTTCTACGTTTAAAGATTCTTGATCTGATC
TTTAAACAAATAGCTAATGATGATTCGCAGGCGACGACGGGGATGCGTGTGACGGATGAGTGTACGAGTTCATTCATGGACATGAAATGGAAGAAAGT
TCATAGATACATCGTTTTCAAGATCGAAGAGAAGTCACGTAAAGTCACCGTCGATAAAGTCGGCGGCGCCGGTGAAAGCTACCACGATCTCGAAGATT
CTTTGCCGGTGGATGATTGTCGCTACGCTGTCTTCGATTTCGACTTTGTCACCGTCGATAACTGCCGCAAGAGCAAGATCTTCTTCATTGCATGGTTA
GTCCAATTTACCTTTATACCCTTCTTAATTCTAGTTGCAAGCAAGGGTAAAACAGTTATTTGATAATATTCGTGCGTCTTAGAAACGCCATATGCCCA
TATAATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAGTGAGTCGTATTAGAGCTTGGCG

b

Figure 6: Computer generated Fasta sequence of the clone (a) pTZPaADF 568 bp putative promoter sequence from *Plectranthus amboinicus* (b) pTZAtADF 766 bp putative promoter sequence from *Arabidopsis thaliana*.

method (InsTAclone PCR Cloning Kit) and the clones were selected based on the blue/white screening method. The presence of white colonies confirmed the presence of the insert within the vector. Clones were further screened for the presence of the insert by colony PCR using M13 primers (forward and reverse) present in the vector. For *Plectranthus*, a fragment of ~650 bp was observed in three clones, and fragments of different sizes (200–1.5 Kb) were observed in *Arabidopsis* [Figure 5a and b]. Fragments longer than 500 bp were selected for further analysis as they likely contain most of the *cis*-regulatory elements of the gene. Among the clones screened, lane 1 had a fragment size of ~600 bp from *Plectranthus* and was named pTZPaADF [Figure 5a] and lane 3 had a fragment size of ~1 Kb from *Arabidopsis* was named pTZAtADF [Figure 5b].

3.5. Sequence Analysis

The resulting clones obtained were sequenced by Sanger's dideoxy method at Agrigenome, Cochin, Kerala using the M13 sequencing primers. Sequence analysis of the clone resulted in 568 bp from *Plectranthus* being named pTZPaADF and 766 bp from *Arabidopsis* being labeled as pTZAtADF [Figure 6a and b]. The sequence of the clone pTZPaADF from *Plectranthus* has been deposited in GenBank under the accession number is OQ280840 (unpublished). The two sequences were subjected to *in silico* analysis for the presence of minimal promoter elements and other *cis*-acting elements.

3.6. *In Silico* Analysis of the Putative Promoter Sequences

The putative promoter sequences were then analyzed using bioinformatic tools such as Ensemble plant database-BLAST, PlantCARE, PLACE, and TSSP softberry. The Ensemble plant BLAST revealed the homology of the two sequences with A. thaliana ADF5 (Gene accession: ADF5 AT2G16700) - for the clone pTZPaADF and pTZAtADF. The sequences were analyzed for similarity using NCBI BLAST, aligning two sequences. 97% similarity was observed between pTZPaADF and pTZAtADF. Plant actin depolymerizing factor binds to both filamentous and globular actin and causes the rapid depolymerization of actin filaments. The identification of the TSS is an important step in analyzing the promoter sequences for their transcription potential. The TSS of the cloned sequences pTZPaADF and pTZAtADF was identified using the TSSPlant a unique tool for the prediction of plant pol II promoters. The transcription start of the clone pTZPaADF was found at 318 bp, with a TSS score of 1.9731, and that of the clone pTZAtADF was found at 223 bp, with a TSS score of 1.9140. The upstream elements (TATA box, CAAT box) necessary for proper transcription were also present in both the putative promoters. From all the *in silico* analysis, it was observed that the clones pTZPaADF and pTZAtADF carried several *cis*-regulatory elements and other essential regulatory motifs which are required for promoter activity. In principle, the gene-specific transcription factors bind to the upstream of the transcription initiation site, which should be rich in AT sequence [21]. Hence, AT percentage of the two sequences was calculated using ENDMEMO. AT % of the clone pTZPaADF was found to be 62% and that of the clone pTZAtADF was found to be 59.39%. Thus, further confirming that the obtained sequences could be potential promoters.

The two putative promoter sequences were analyzed for the presence of *cis*-regulatory 'elements using the PlantCARE and PLACE databases. The database identified many general transcription regions and potential regulatory elements of the two sequences are summarized

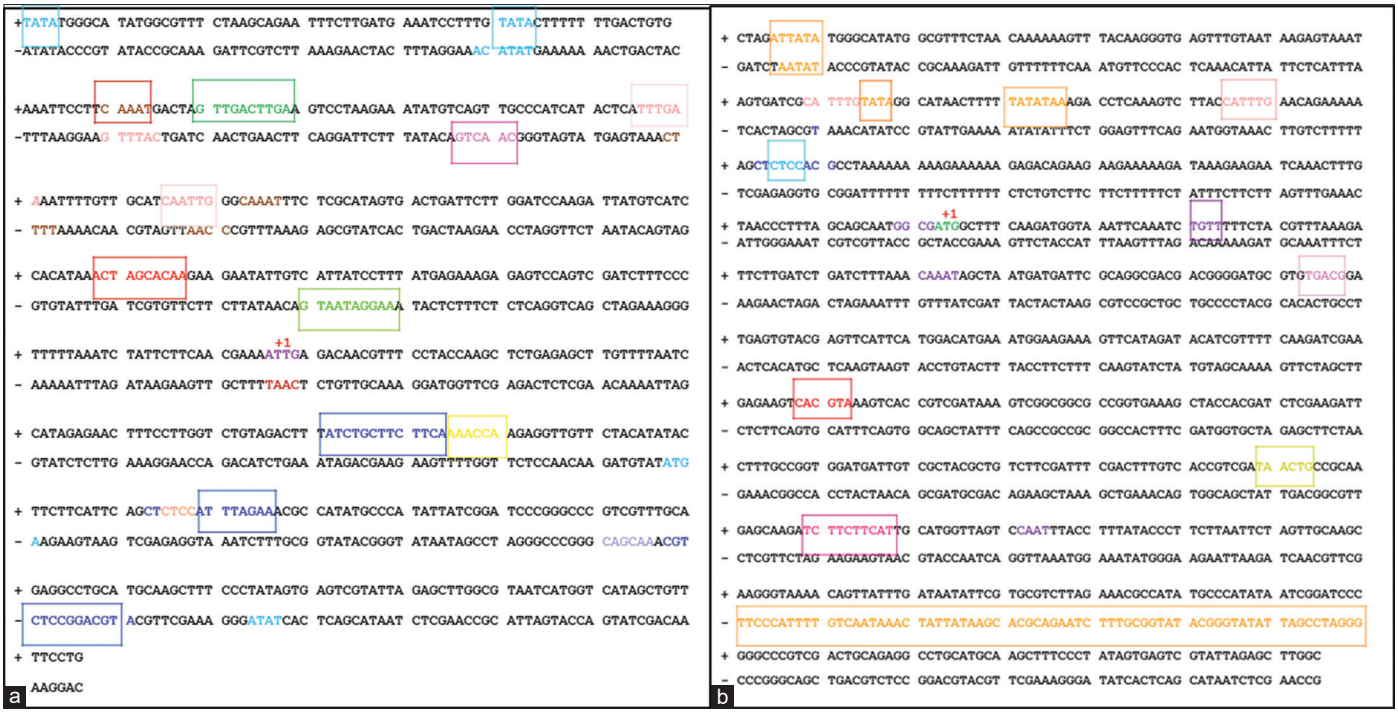

Figure 7: (a) pTZPaADF putative promoter sequence of *Plectranthus amboinicus*. A schematic representation showing various cis elements as determined by Plant CARE database. ARE CAAT CTAG motif GATA motif MBS MYC Myb O2site TATA box TGA element +1- Transcription start site. (b) pTZAtADF putative promoter sequence of *Arabidopsis thaliana*. A schematic representation showing various cis elements as determined by Plant CARE database. ABRE ABRE3a ABRE4 AT ~ TATA- box CAAT box CGTCA motif DRE core -box G-box MYB Myb like sequence Myb TATA box TCA +1- Transcription start site.

in in Table 3 (PlantCARE) pTZPaADF, Table 4 (PlantCARE) pTZAtADF and Table 5 New PLACE for the clones. The analysis revealed the presence of several *cis*-acting elements such as the TATA box, CAAT box, and G-box which are essential for promoter activity in both sequences [22]. The TATA box is important for binding the other general transcription factors as well as choosing the precise transcription start point. The CAAT box, which is typically present between −80 and −150 bp upstream of the TSS, is crucial for the binding of transcription factors [23]. The outcomes are similar to the previous study reported in Arabidopsis, Rice, Pisum, and Tobacco [24]. The following motifs were observed in the clone pTZPaADF such as: ARE motif at +395 bp position (Anaerobic induction); light responsive elements: CTAG motif at +218 bp, GATA motif at -240 bp. The most studied element for drought resistance is the MBS, the Myb binding site that is present in the −117 bp position. Hormone-responsive elements (TGA element - Auxin, responsive element at −481 bp, TCA - salicylic acid responsiveness at −227 bp, O2 site - Zein metabolism at +90 bp were also found to be present [25]. *In silico* analysis of the putative promoter pTZAtADF from *Arabidopsis* also showed significant *cis*-regulatory elements for promoter activity. ABRE, *cis*-acting element involved in the abscisic acid responsiveness was found at −428 bp, DRE core, Drought resistant element was found at −451 bp. The *cis*-acting regulatory element involved in light responsiveness G- box was found at −428 bp. Hormone-responsive elements such as methyl jasmone responsiveness [26], CGTCA-motif, and MYB are present at +344 bp and −583 bp, respectively. All the cis-acting elements from both the putative promoters are listed in Tables 3 and 4.

The schematic representation of the *cis*-elements for pTZPaADF and pTZAtADF is given in Figure 7a and b, respectively. The putative promoter sequences were subjected to computational analysis, which

Table 3: Identified *cis* acting elements and their positions of pTZPaADF putative promoter from the PlantCARE database.

cis elements	Sequence	Position	Strand	Functions
ARE	AAACCA	395	+	*cis*-acting regulatory element essential for the anaerobic induction
CAAT-box	AATCTAATCT	235	-	Common *cis*-acting element in promoter and enhancer regions
CTAG-motif	ACTAGCAGAA	218	+	
GATA-motif	AAGGATAAGG	240	-	Part of a light responsive element
MBS	CAACTG	117	-	MYB binding site involved in drought-inducibility
MYC	CAATTG	155	+	Cellular functions
Myb	CAACTG	117	-	Stress response
O2-site	GTTGACGTGA	90	+	*cis*-acting regulatory element involved in zein metabolism regulation
TATA-box	TATA	416	-	core promoter element around−30 of transcription start
TGA-element	AACGAC	481	-	Auxin responsive element
Unnamed__4	CTCC	435	+	Unknown
TCA-motif	GAGAAGAAT	227	-	*cis* acting element involved in salicylic response
TGA element	AACGAC	482	+	Auxin responsive element

Table 4: Identified *cis* acting elements and their positions of pTZAtADF putative promoter from PlantCARE database.

cis elements	Sequence	Position	Strand	Functions
ABRE	ACGTG	428	-	*cis*-acting element involved in the abscisic acid responsiveness
ABRE3a	TACGTG	428	-	
ABRE4	CACGTA	428	+	
AT~TATA-box	TATATA	101	+	Common *cis*-acting element in promoter and enhancer regions
CAAT-box	CAAAT	126	-	Common *cis*-acting element in promoter and enhancer regions
CGTCA-motif	CGTCA	344	+	*cis*-acting regulatory element involved in the MeJA-responsiveness
DRE core	GCCGAC	451	-	Drought resistant element
G-box	TACGTG	428	-	*cis*-acting regulatory element involved in light responsiveness
MYB	TAACCA	583	-	*cis*-acting regulatory element involved in the MeJA-responsiveness
MYB-like sequence	TAACCA	583	-	Core promoter element around−30 of transcription start
MYC	CATTTG	125	+	*cis* acting element involved in salicylic response
Myb	TAACTG	549	+	Part of a light responsive element
TATA-box	ccTATAAAaa	152	+	Core promoter element around−30 of transcription start
TCA	TCATCTTCAT	569	+	
TCT-motif	TCTTAC	119	+	Part of a light responsive element
TGACG-motif	TGACG	344	+	*cis*-acting regulatory element involved in the MeJA-responsiveness

Table 5: List of motifs, their position and predicted function using New PLACE of pTZPaADF and pTZAtADF.

S. No.	Motif name/Factor	Starting position from ATG pTZPaADF	Starting position from ATG pTZAtADF	Sequence	Predicted function
1.	EBOXBNNAPA	9	15	CANNTG	E-box of napA storage-protein gene of Brassica napus (B.n.); This sequence is also known as RRE (R response element)
2.	CAATBOX1	158	601	CAAT	CAAT promoter consensus sequence
3.	ARR1AT	42	3	NGATT	"ARR1-binding element" found in Arabidopsis; ARR1 is a response regulator
4.	GT1CONSENSUS	246	186	GRWAAW	Consensus GT-1 binding site in many light-regulated genes
5.	IBOXCORE	247	192	GATAA	"I box"; "I-box"; Conserved sequence upstream of light-regulated genes.
6.	DOFCOREZM	252	209	AAAG	Core site required for binding of Dof proteins.
7.	MYBST1	248	551	GGATA	Core motif of MybSt1 binding site.
8.	TAAAGSTKST1	252	454	TAAAG	TAAAG motif found in promoter of Solanum tuberosum (S.t.) KST1 gene; Target site for trans-acting StDof1 protein controlling guard cell-specific gene expression.
9.	MYCCONSENSUSA T	459	127	CANNTG	MYC recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes Arabidopsis.
10.	WBOXNTERF3	558	432	TGACY	"W box" found in the promoter region of a transcriptional repressor ERF3 gene in tobacco; May be involved in activation of ERF3 gene by wounding

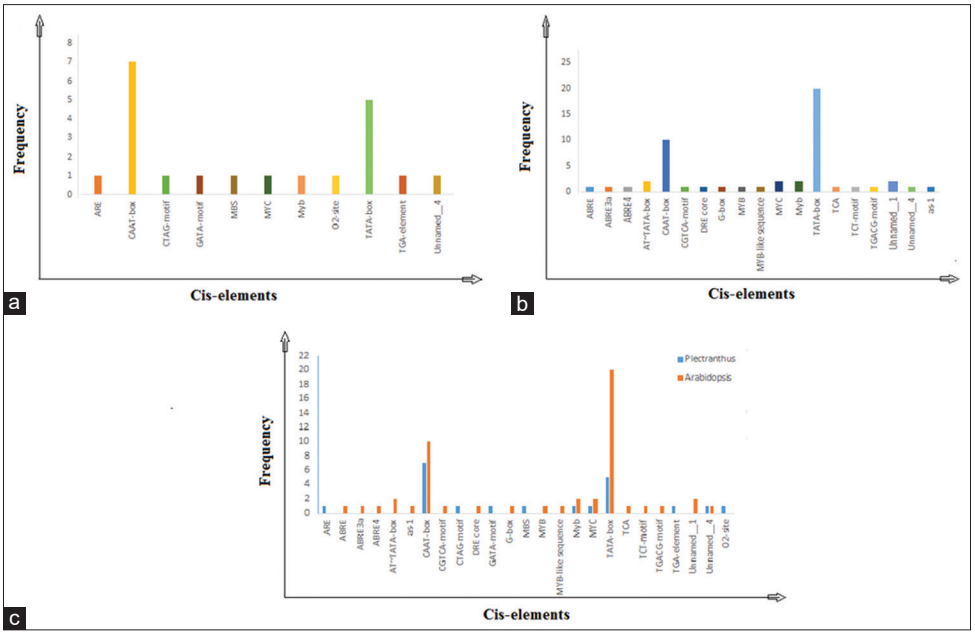

Figure 8: Frequency of motifs identified using PlantCARE in the actin depolymerising factor putative promoter regions of (a) *Plectranthus* (b) *Arabidopsis* (c) Both.

revealed *cis*-acting regions with notable homologies to the elicitor, transcription factors, and hormone-responsive elements. Motifs involved in stress-responsive were also found to be present in the putative sequences. Some were present in both the sequences while some were limited only to *Arabidopsis* are discussed in Figure 8. The New PLACE tool showed the presence of various stress-responsive and hormone-regulation motifs common to *Plectranthus* and *Arabidopsis* are listed in Table 5. Very few motifs like O2 site Skn-1 involved in cellular response were also found to be present. All the biologically functional motifs in pTZPaADF and pTZAtADF are shown in Figure 9a and b, respectively. The predicted *cis*-acting elements in the pTZPaADF from *P. amboinicus* and pTZAtADF from *A. thaliana* putative promoter region have been reported for the first time.

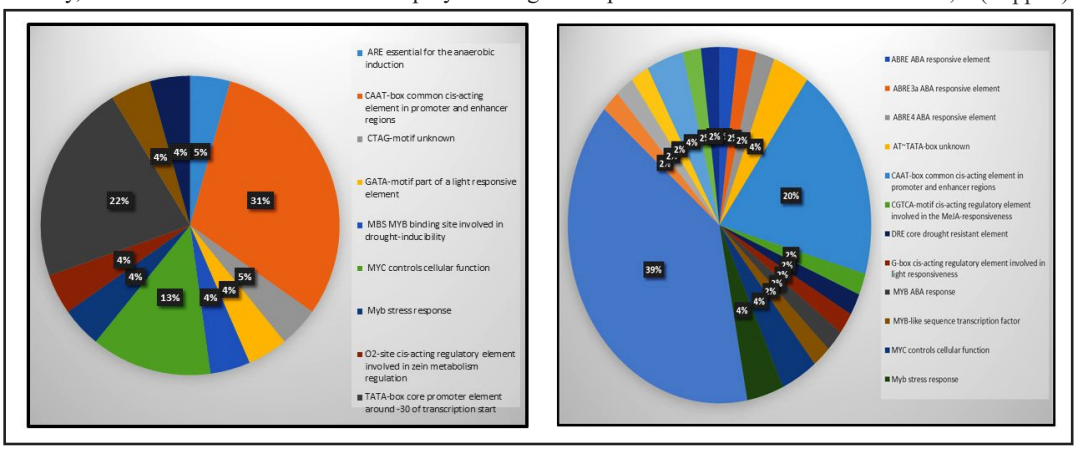

Figure 9: Pie distribution of identified motifs of (a) pTZPaADF from *Plectranthus* and (b) pTZAtADF from *Arabidopsis* from PlantCARE based on their biological functions.

All transcription factors and RNA polymerases can attach to the promoter sequence and control the gene's expression. *cis*-regulatory elements are a particular DNA sequence that transcription factors will detect and use to control the gene expression under various circumstances [27]. In plants, the first ADF was described in *Lilium longiflorum* (lilies) where the screening of clones that were expressed in anthers. Existing reports suggests that the ADFS are also associated in signalling defense mechanism and disease resistance in the plant [28,29]. The availability of genome sequences of many plant species including the model dicot *A. thaliana* and accessibility to the wide availability of web-based genome browsers and databases have opened up ways to investigate gene families in detail. In *A. thaliana* 11 members of ADFs were described that are manifested as tissue, organ-specific, and constitutive gene expression patterns [30] particularly distinct expression patterns were in AtADF 1, At ADF 5, and AtADF6 in root apical meristem [31].

From the previous studies, it is evident that ADF is involved in various aspects of plant physiology. Understanding the mechanism and control of plant actin depolymerizing factor and its use in plant transgenesis can be greatly aided by the findings. The findings from the study together with evidence obtained from *A. thaliana* suggest that the sequence pTZPaADF is found to be homologous and conserved among various plant species. Based on the *in silico* analysis, all the essential upstream elements needed for promoter activity were present in pTZPaADF and pTZAtADF and could be applied in plant genetic transformation studies.

4. CONCLUSION

The current study successfully sequenced the putative promoter region of plant ADF from *P. amboinicus* using *A. thaliana* as an internal reference. TAIL PCR was used to isolate the 5' region of the plant ADF gene from both plants, demonstrating the applicability of the method to isolate promoter regions from plants. Furthermore, *in silico* analysis showed that the putative promoters pTZPaADF and pTZAtADF possess critical *cis*-regulatory elements that could potentially drive and control transgene expression under various conditions. However, further studies are needed to functionally validate the isolated pTZPaADF and pTZAtADF promoter activities by plant transgenic methods and gene expression studies using appropriate reporter genes.

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All data generated and analysed are included with this research article.

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