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Isolation and *In-Silico* Analysis of A Novel *Cucumis sativus* Polyubiquitin Gene Promoter

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ABSTRACT

Transcriptional regulation is a dynamic process and the first level of gene expression control, largely governed by the gene promoters and their contributing cis-regulatory elements (CREs), the binding of which to the regulatory proteins and transcription factors (TFs) lead to the activation or repression of the genes. Strong constitutive promoters that drive higher expression levels have become a valuable tool in the genetic engineering of plants. Recently, the search for strong plant-derived constitutive promoters has expanded to several monocot and dicot species. In this study, we isolated a novel promoter of ~850 bp located in the upstream of a house keeping *Cucumis sativus* polyubiquitin gene (Ub) with an open reading frame (ORF) of 1374 bp. The conserved domain results of the Ub gene revealed the presence of a typical Ub like superfamily conserved domain and a k27 lysine residue involved in the chain linkage of Ub genes. The bioinformatics analysis of the cis-regulatory elements (CREs) using plant CARE and PLACE showed 22 functional classes in the Ub gene promoter, many of which are involved in the binding of transcription factors (TFs) for the expression during abiotic and biotic stresses. Therefore, it would be an ideal choice of plant-derived promoter for driving higher levels of transgene expression in dicots especially in cucurbits which needs further validation by gene expression and functional characterization studies.

Keywords : Cucumis sativus, CREs, *In silico*, Polyubiquitin, Promoter analysis

GENE promoters are the regulatory sequences located upstream of the gene coding regions involved in the transcriptional expression of the gene. They contain multiple cis-regulatory elements (CREs); their nature and organization has a major impact on the promoter strength and determines the specificity of binding to the trans-regulatory proteins; transcription factors (TFs) required for the initiation and regulation of the transcription process (Ho and Geisler, 2019).

The crop genetic improvement through 'green technology approaches' such as genetic engineering and genome editing techniques *via* TALENS, ZFNs and CRISPR require a strong and well-regulated promoter for driving higher expression levels of the foreign genes or utilization of cis-genic elements. Several types of promoters such as constitutive, inducible, tissue-specific, viral and synthetic are used in the plant genetic transformation. A constitutive promoter is the one which is able to drive gene expression in many or all tissues or throughout most of the lifecycle of the plant (Kummari *et al.,* 2020).

The most widely used is a viral based promoter Cauliflower Mosaic Virus (CaMV) 35S for directing strong constitutive expression in transgenic plants (Porto *et al.,* 2014). Apart from CaMV35S, peanut chlorotic streak caulimo virus (PC1SV) and figwort mosaic virus (FMV) have also been shown to be very useful for generating Genetically Modified (GM) plants. Previous research findings show evidence of the increased chances of transcriptional inactivation due to the overuse of CaMV35S promoter (Chen *et al.,* 2013). An earlier report has shown the silencing of bar transgene, a commercially important genetic trait in transgenic oilseed rape leading to the altering of the plant phenotype from herbicide resistance to susceptibility upon CaMV infection (Bak and Emerson, 2020). It is also not uncommon to find in the literature that CaMV 35S promoters used to drive two or more

chimeric genes in the same transformation vector. The CaMV 35S also gives rise to the gene silencing phenomenon. Therefore, to avoid the potential risk of gene-silencing associated with CaMV35S and to introduce multiple transgenes, it is important to isolate and characterize a wide range of novel plant-derived promoters driving higher expression in plants.

In monocot species, certain constitutive promoters of plant origin, such as rice actin promoter and maize ubiquitin promoter (Beringer *et al.,* 2017), have been isolated and are often used for transformation of grasses (Wang *et al.,* 2016). In dicot plants, although a number of endogenous constitutive promoters have been isolated, they are not widely used or tested in other species, particularly in legumes. Although a number of constitutive promoters have been isolated from plants and used for the generation of transgenic plants, there is still a great need for novel plant sequences that function as promoter elements for the high-level expression of transgenes.

With the availability of genome sequence information (Huang *et al.,* 2009) and development of genetic transformation protocols in the model plant *Cucumis sativus*, it is essential to explore, isolate and characterize several endogenous promoters to drive higher level of transgenic expression. With this in the view, we isolated a novel promoter from a regulatory polyubiquitin gene (Ub) from *C. sativus* and identified the presence of multiple CREs through bioinformatics analysis with the possibility of utilizing them as a constitutive promoter to drive higher levels of expression of genes under biotic and abiotic stresses. Further, functional characterization and gene expression studies are required to validate for its application as a strong promoter to drive high levels of transgene expression in cucurbits.

Material and Methods

Sample Collection and Extraction of Plant Genomic DNA

The *Cucumis sativus var* green long plants were maintained in the greenhouse under standard conditions and the leaf samples were collected for the genomic DNA isolation. The modified SDS protocol was adopted for the genomic DNA isolation and the pellet was air dried and 0.1X $T_{10}E_1$ buffer was added and stored at -20 °C until further use (Sahakar & Peter, 2015; Tak & Peter, 2016 and Xia *et al.*, 2019).

Quantity and Quality Assessment of the Genomic DNA

Isolated genomic DNA was quantified by Nanodrop spectrophotometer (Bio Spectrometer, Eppendorf, Germany). About 1-2 µl of DNA sample was kept in the nano drop spectrophotometer and the absorbance was read at 260 nm. An Optical Density (OD) of 1 at 260 nm correlates to a double stranded DNA concentration of 50 ng/µl. Assessment of DNA quality was carried out by resolving the genomic DNA using 0.8 per cent agarose gel electrophoresis, stained with ethidium Bromide (EtBr) and visualized under UV light. The purity and presence of intact DNA was checked prior to PCR analysis.

Retrieval of Upstream Sequences of Polyubiquitin Gene and Primer Designing

The sequences (1kb located upstream of the translation start site) of poly ubiquitin gene (Ub) (CsaV3_6G049240) was retrieved from the Cucurbit genomic database (http://cucurbitgenomics.org). The gene specific forward and reverse primers were designed using Primer3 tool (v. 0.4.0) and the following forward CP: 5' CATGTCCGTCTCGCTATCGTCT CCCAAACTCTAACA -3') and reverse CP 5' - CATGTCCGTCTCGatTCTGGAAGACAAAGGATTAGG -3'), respectively adhering to the basic principles governing the primer design**.**

PCR Amplification Conditions

The isolated genomic DNA was amplified using Ub promoter specific forward and reverse primers. The reaction was set up in a 25 µL final volume containing 25-30 ng DNA template, 14 µL deionized nuclease free water, 2.5 µL 10X PCR buffer with 15 mM MgCl2, 2.5 µL 2 mM dNTPs, 1.0 µL forward and reverse primer (each of 10 pmol/µL) and 1.0 µL *Taq* DNA

polymerase (1U/µL) (3B Biotech, India). The amplification was carried out using a GeneAmp PCR system 9700 thermal cycler (Bio-Rad laboratories, USA) with the following amplification conditions of initial denaturation at 94 ºC for 4 minutes, denaturation at 94 ºC for 45 seconds annealing at 60 ºC for 1 minute, extension at 72 ºC for 1 minute 30 seconds and final extension at 72 ºC for 8 minutes with 32 cycles of amplification.

Analysis of the PCR Products

The resulting PCR products (10µL) were mixed with 1.5µL of 6x loading dye (30 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue and 0.25 % (w/v) xylene cyanol FF) and separated electrophoretically in 1 % agarose gel (3B low EE agarose) with 1X Tris Acetate EDTA (TAE) buffer (50x buffer: 242g Tris base, 57.1mL glacial acetic acid, 100mL 0.5M EDTA (pH 8.0) and dH_2O) along with 3 µL of 100bp standard size DNA ladder (Thermos Scientific, India) and run at 80v for 2 hours (Genei, Bangalore, India). Gel was stained with EtBr and the bands were visualized under UV A_{260}nm (Alpha Innotech, FlourChem SP imaging system, USA) and the banding pattern was observed. The amplicons were further purified according to manufacturer's instruction for sequencing (Thermos Scientific, India).

DNA Sequencing

Sequencing of the purified amplified product was done at a commercial facility (Scigenomics, Kerala, India). Both the forward and reverse sequences were assembled and aligned using Bio Edit sequence alignment software (v 7.1) followed by a similarity search with the BLAST feature in the Cucurbit genomics database.

Structural Analysis of Polyubiquitin Gene

The conserved domain of the Ub gene sequence retrieved from Cucurbit genomics database (Accession number: CsaV3_6G049240) was predicted using NCBI domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer *et al.,* 2017).

Analysis of CIS - Regulatory Elements (CREs**) of Ub Promoter**

The cis elements of *C. sativus* Ub promoter was predicted using the tools Plant Care (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/).

Results and Discussion

BLAST Analysis

Amplification of the isolated genomic DNA with Ub promoter specific primers yielded an amplicon size of ~900 bp (Fig. 1). The sequenced amplicon was subsequently analyzed in the cucurbit genomics database using the BLAST feature and the results revealed a sequence identity of 99.8 per cent with the scaffold02364 of *C. sativus* green long (Gy14) v2 genome, confirming it as the Ub promoter (http://cucurbitgenomics.org/blast). The sequenced promoter was submitted to NCBI with an assigned accession number: MN243922.1.

Structural Analysis of Ub Gene

The structural analysis of Ub gene showed the presence of a conserved domain; ubiquitin-like-fold superfamily domain characteristic of ubiquitin proteins and also a key conserved k27 amino acid residue; one of seven lysines involved in chain linkage in ubiquitin (K6, K11,

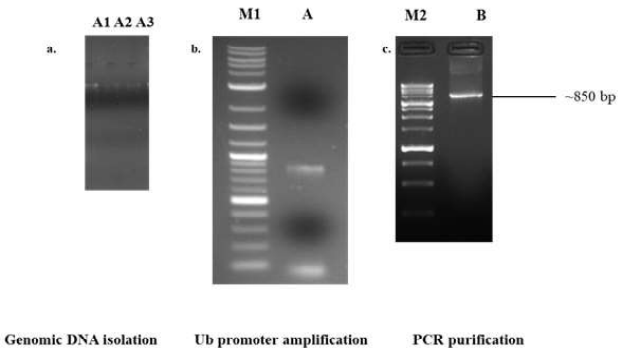

Fig. 1: The agarose gel electrophoretic images of a) *C.sativus* genomic DNA isolation, lanes A1, A2 and A3 specifying the isolated genomic DNAs. b) Polyubiquitin gene promoter, lane M1and M2- 100 bp DNA ladder (Thermo Scientific, India) and A- Ub promoter amplicon and c) PCR purified Ub promoter amplicon

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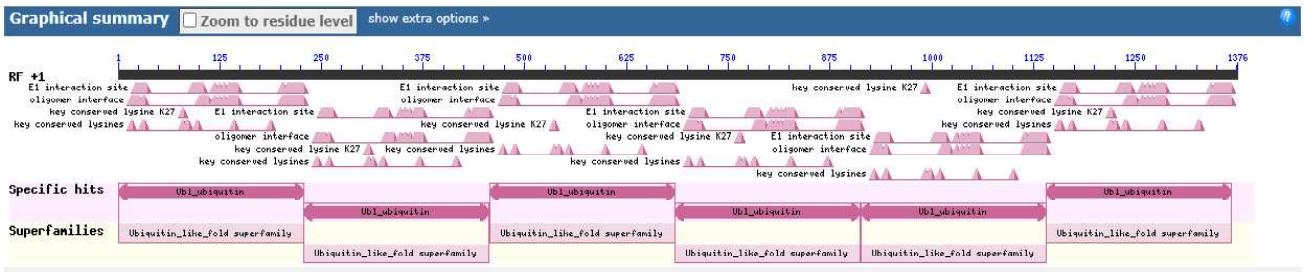

Fig. 2 : The conserved domains of *C. sativus* Ubi gene using CDD NCBI search

K27, K29, K33, K48 or K63, Ub numbering (Fig. 2). Ubiquitin is a protein modifier in eukaryotes that is involved in various cellular processes, including transcriptional regulation, cell cycle control, and DNA repair. Ubiquitination is comprised of a cascade of E1, E2 and E3 enzymes that results in a covalent bond between the C-terminus of ubiquitin and the epsilon-amino group of a substrate lysine (Tracz and Bialek, 2021). Ubiquitin-like (Ubl) proteins have similar ubiquitin beta-grasp fold and attach to other proteins in an Ubl manner but with biochemically distinct roles. Ubiquitin (Ub) and Ubl proteins conjugate and deconjugate *via* ligases and peptidases to covalently modify target polypeptides. Ub includes Ubq/RPL40e and Ubq/RPS27a fusions as well as homo polymeric multiubiquitin protein chains (Yi *et al.,* 2017). Our results were in accordance with the previous findings confirming the presence of Ub conserved domains in the polyubiquitin gene (Ub).

The analysis revealed a total of 22 Cis-regulatory elements (CREs) in the Ub gene promoter. The length of the CREs varied from 4 to 10 bp with an average range of 5-6 bp length in majority. The CREs were classified into different functional groups based on the data obtained and are summarized in Table 1. The functional classes were categorized into groups which included CREs involved in the normal cellular development process, stress responsiveness, hormonal regulation and some reported with unknown function. Majority of the CREs were observed in the 44-674 bp regions on the forward strand and 130-778 regions in the reverse strand. The CREs TATA and CAAT elements involved in the normal transcriptional control of gene were in predominantly higher frequencies covering about 61 per cent than the other functional classes. The role and importance of CREs involved in different functional classes are discussed below.

CREs in Stress Response

The CREs for stress response occupied 17 per cent of the promoter gene sequences comprising anaerobic induction response and light response elements (Fig. 3). AREs are Anaerobic Responsive Elements with AAACCA motif sequences (Table 1). The presence of AREs in the promoter region is known to play a significant role in acclimation to various stresses including oxidative and flooding stress. The interaction of AREs and transcription factors (TFs) is reported to regulate the transcriptional levels of genes involved in ATP production through the fermentation pathway during anaerobic energy metabolism under low oxygen concentrations. Similarly, both maize and *Arabidopsis* ADH1 promoters have a bipartite ARE element consisting of GT- and GC-motifs, which are crucial for gene expression under stress conditions (Kaur and Pati, 2016).

There are several light responsive elements present in the Ub gene promoter sequences including G-Box with 'CACGTT' sequence motifs, I-box with

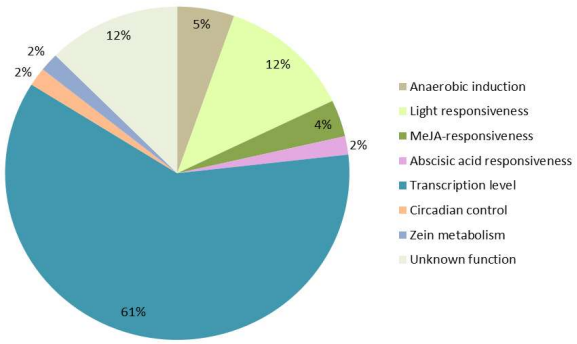

Fig. 3 : The pie chart distribution of various CREs of the *C. sativus* Ub gene according to their functional classes

TABLE 1

Details of the CREs extracted from plant CARE database for the *C. sativus* Ub gene promoter

Motif/ Cis element	Sequence	Motif function
ARE	AAACCA	Cis-acting regulatory element essential for the anaerobic induction
CGTCA-motif	CGTCA	Cis-acting regulatory element involved in the MeJA-responsiveness
TCA	TCATCTTCAT	-
CAAT-box	CAAAT	Common cis-acting element in promoter and enhancer regions
Unnamed__1	CGTGG	-
TATA-box	ATATAA and TATA	Core promoter element around -30 of transcription start
G-Box	CACGTT	Cis-acting regulatory element involved in light responsiveness
ERE	ATTTCATA	-
MYB-like sequence	TAACCA	-
ABRE	ACGTG	Cis-acting element involved in the abscisic acid responsiveness
MYB	TAACCA	-
Circadian	CAAAGATATC	*Lycopersicon esculentum* cis-acting regulatory element involved in circadian control
MYC	CATTTG	-
I-box	gGATAAGGTG	Part of a light responsive element
GATA-motif	AAGGATAAGG	Part of a light responsive element
STRE	AGGGG	-
AS-1	TGACG	-
AE-box	AGAAACT	Part of a module for light response
AAGAA-motif	GAAAGAA	-
TGACG-motif	TGACG	Cis-acting regulatory element involved in the MeJA-responsiveness
WRE3	CCACCT	-
O2-site	GATGA(C/T)(A/G)TG(A/G)	Cis-acting regulatory element involved in zein metabolism

gGATAAGGTG, GATA-motif AAGGATAAGG and AE-box AGAAACT modules respectively. Literature evidences suggest multiple roles of G-box elements in response to light, abscisic acid, methyl-jasmonate and anaerobiosis and also in ethylene induction as well as in seed specific expression (Ezer *et al.,* 2017). Earlier reports suggest the contribution of strong expression levels of Ub gene promoter mediated by a well conserved G-box like motif in the soybean Gmubi3 and Gmubi7, rice RUBQ2 andrubi3, maize Zmubi1, sunflower UbB1, switchgrass PvUbi1, and potato Ubi7 promoters (Liu *et al.,* 2016). Similarly, the occurrence of conserved tetramers of this element (ACGT) is observed in the isolated Ub promoter, suggesting their possible role in high expression levels.

CREs in Hormonal Regulation

CREs of this class contributes to 8 per cent of the total CREs with ABA, methyl jasmonate, and zein hormonal response elements. The results revealed the presence of a typical consensus ABRE motif ACGTG, which is also found in other species including rice and *Arabidopsis* that regulates dehydration and salinity responses. The ACGT core, characteristic of these cis-acting DNA elements is known to interact with a group of basic leucine zipper (bZIP) transcription factors for various physiological responses of many ABA-regulated genes in plants (Sarkar and Lahiri, 2013). Presence of another well characterized conserved motif sequence CGTCA involved in the

Me-JA response was also observed. The jasmonic acid (JA) and its derivatives (JAs) serve as signaling molecules to regulate diverse aspects of plant life including leaf senescence, tuber formation, tendril coiling and filament elongation, biotic and abiotic responses (Wang *et al.,* 2011). Similar observations were made in the promoter sequences of JAZ family genes with several conserved motifs, such as G-box (CACGTG) and CGTCA-motif (CGTCA) related to jasmonate signaling. Similarly in *Poncirus trifoliate*, the promoter element of PtAO (Allene oxide) gene involved in JA biosynthesis typically comprised a CGTCA-motif element involved in MeJA responsiveness (Xiong *et al.,* 2020). The presence of another hormonal regulating Opaque2 (O2) motif sequences recognizing basic leucine (Leu)-zipper transcriptional activator controlling the gene expression of zein metabolism was also seen in the upstream Ub promoter sequences (Zhang *et al.,* 2015).

CREs in Cellular Development and Possible Role of CREs with unknown Function

The presence of CAAAGATATC motif involved in the circadian regulation accounted for 2 per cent of the total CREs. In addition to the other functional classes, there were also cis-elements with unknown function which constituted 12 per cent of the total CREs present in *C. sativus* Ub promoter region. Various elements such as ERE, Myb, Myc, STRE and as-1 element were observed. Their contribution in the plant stress and the relevant literature reported previously are discussed below.

The role of STRE elements in the regulation of gene expression during heat-stress conditions is reported in several research studies. For example: In Arabidopsis, it was demonstrated through deletion assay of AtHsp90–1 gene promoter. Similarly, it is also reported to occur as one of the heat shock element in Heat Shock Factor HsfA1a. An earlier study identified RSRE cis-elements (AAGGGG) resembling STRE in the promoters of diverse rapid wound responsive genes in Arabidopsis and functionally involved in stress responses, which is similar to the AGGGG motif sequence identified in this study. AGGGG (STRE) is a binding site for transcriptional activator, Msn2p/ Msn4p, identified in yeast and responsive to various stresses and also upon elicitor induction in wounding (WUN-motif, WRE3 and box S) (Montibus *et al.,* 2015).

The role of ethylene responsive elements (ERE), as a potential tool for rapid high-throughput analysis of in planta pathogen responses is previously reported (Hernandez-Garcia and Finer, 2014). Functional studies have shown that MYB is involved in plant secondary metabolism, hormone and environmental factor responses and plays an important regulatory role in cell differentiation, cell cycle and leaf morphogenesis (Sheshadri *et al.,* 2016 and Li *et al.,* 2019). The regulation of the expression of *CBF3*/ *DREB1A* by the binding of MYC-like bHLH (basic-helix-loop-helix) transcription factor in the canonical MYC *cis*-elements (CANNTG) is previously reported, the presence of such canonical sequences were also observed here with CATTTG motifs.

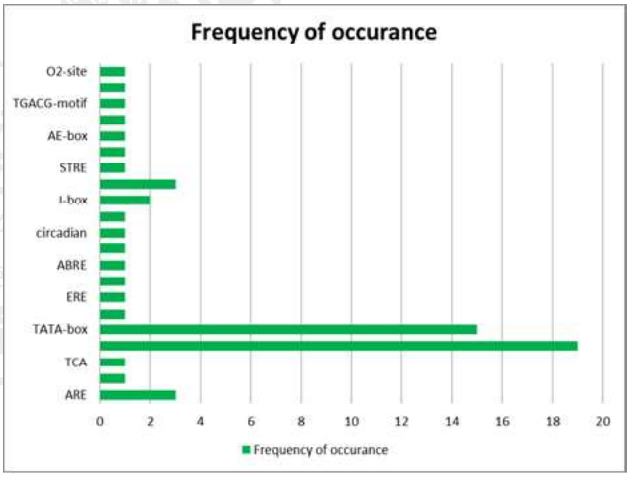

Fig. 4 : The frequency of the motifs found in the *C.sativus* Ub promoter using PLantCARE database.

Bioinformatics analysis of the upstream promoter elements comprising MYB and MYC elements along with ABRE (ABA responsive element), DRE/CRT (dehydration-responsive element/C-repeat, and the E-box elements have shown to exhibit a functional role possibly involved in the chilling or cold responses across different plant species (Ohta *et al.,* 2018).

Analysis of the promoter sequence alignments in peas (GCG 8.0, Genetics Computer Group, Madison, WI)

revealed three regions of identity namely WRE1 (wound-response element 1, AAATTTC motif), WRE2 and WRE3 (CCACCT) that are potentially involved during the common wound induction of the *CYP73A9v1* and *CYP82A1v1*. The occurrence of such WRE3-like CRE was observed in the upstream Ubi promoter sequence. The TGACG-box (TGACG) popularly known as 'as-1 motif', is another well characterized cis-element present in plants. TGACG motif is methyl jasmonate responsive element present among *A. thaliana* and *O. sativa* PR gene sequences (Wang *et al.,* 2012).

Promoters play an essential role in initiating and regulating the transcription, the first and the most important step of gene expression, their isolation and functional characterization is therefore important. High-expressing housekeeping genes that encode abundant proteins required for basic functions in plant cells are a good source of strong native plant-derived constitutive promoters. The endogenous promoter isolated in this study could emerge as a promising candidate promoter for genetic manipulation of abiotic and biotic stress tolerance in crops for high-level expression of transgenes after a thorough functional characterization and expression analysis studies.

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