The Pharma Innovation Journal 2022; 11(9): 2063-2067

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(9): 2063-2067
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www.thepharmajournal.com
Received: 13-06-2022
Accepted: 30-07-2022

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Cloning and *in silico* analysis of the upstream region of an endodermis specific promoter in rice

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Abstract
SCARECROW (SCR) is a plant-specific transcription regulator that is well-known for its role in stem-cell regeneration in plant roots. SCR is expressed in the initial cells of the cortex/endoderm and the endodermal cell lineage. Tissue specific expression of SCR is regulated at the transcriptional level. In this study a 1.29 kbp upstream region of the SCARECROW gene was demarcated and analyzed for the Cis-regulatory elements and transcription start site (TSS). Through PCR the 1.29 kbp was cloned using specific primers from the rice genotype cv. *Mundan*, a salt tolerant landrace. Analysis of potential *cis*-elements using Plant CARE database deduced 24 *cis*-regulatory elements and one TSS.

Keywords: Cloning, *in silico*, upstream, endodermis, promoter, rice

Introduction
Scarecrow (SCR) is a member of the plant-specific GRAS family transcriptional regulators which plays a crucial role in stem cell maintenance and radial patterning in the roots (Di Laurenzio *et al.,* 1996) [5]. The ground tissue forms two layers *viz.,* the endodermis and cortex, by asymmetric cell division of the cortex/endodermis initial (CEI) cells. Anticlinal division of the cortex/endodermal initial generates two cells with distinct developmental potentials in the initial stage. One will continue to serve as an initial, while the other will undergo Periclinal division to produce the first cells in the endodermal and cortical cell lines. The SCR is initially expressed in both the cortex and endoderm, but after cell division, expression is confined to the endodermal lineage due to differential transcriptional regulation. The *scr* mutant lacks this second asymmetric division, resulting in a single cell layer as opposed to two (Ogasawara *et al.,* 2011) [12]. Di Laurenzio *et al*. (1996) [5] found that the single cell layer in *scr* mutant has both cortical and endodermal features. Further, in the *scr* mutant, quiescent center (QC) and the stem cell niche (SCN) was absent. SCN is made up of cells that surround the QC and have stem cell characteristics that give rise to different cell types *viz*., cortex and endodermis (Benfey *et al.,* 1993; Sabatini *et al.,* 2003) [1, 13].
SCR transcription factor is built by 653-amino acids, which contains many functional domains. A charged region between residues 265 and 283 amino acids is identical to the basic domain of the basic-leucine zipper (bZIP) family of transcriptional regulatory proteins (Hurst, 1994). Essentially, short-root (SHR), and SCR are two transcription factors that are crucial for determining the cell fates of the ground tissue's cortical and endodermal layers. SHR is transcribed in the immature vasculature (Helariutta *et al.,* 2000) [8] but SCR is transcribed in to endodermis (Helariutta *et al.,* 2000; Di Laurenzio *et al.,* 1996) [8, 5]. SCR and SHR also found to be express in plant shoots (Dhondt *et al.,* 2010; Cui *et al.,* 2014) [4, 3]. SCR is localized in vascular tissue while SHR in bundle sheath (BS) cells. To interact with SCR, SHR enters bundle sheath cells and travels into the nucleus, in a similar way it interacts in roots (Cui *et al.,* 2014) [3]. Recent studies in *Arabidopsis thaliana* and *Zea mays* showed that, BS cell fate and function are regulated by both SCR and SHR (Cui *et al.,* 2014; Gao *et al.,* 2014; Slewinski *et al.,* 2012) [3, 6, 14]. By controlling cell proliferation, SCR and SHR also control leaf growth (Dhondt *et al.,* 2010) [4]. The SHR protein is translocated to the cortex-endodermal initial (CEI), where it interacts with SCR to promote asymmetric cell division *via* a positive feedback loop (Heidstra *et al.,* 2004; Helariutta *et al.,* 2000; Nakajima *et al.,* 2001) [7, 8]. Recent study demonstrated that SCR is necessary for endodermal specification *via* acting redundantly with SCL23, a near homolog of SCR (Long *et al.,* 2015) [10]. Given the endodermis specific expression of SCR, here we have made an attempt to clone and analyze the upstream region of

the *SCR* gene towards developing an endodermis specific promoter for use in genetic engineering of rice crop for abiotic stress tolerance.

Materials and Methods

A 1.29 kbp upstream region of the *SCR2* gene (LOC_Os12g02870) falling on the chromosome 12 of *Oryza sativa Japonica* Group *cv*. Nipponbare was demarcated for cloning and *in silico* analysis. The above region was retrieved from GenBank, NCBI which encompassed the putative promoter of *SCR2* gene and a portion of the 5'- UTR. *In silico* analysis of upstream sequence was performed using two different online tools *viz.,* i) Plant CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), ii) Transcription start site was predicted by using TSS Plant (http://www.softberry.com/berry.phtml).

PCR based cloning of upstream sequence of *SCR2* gene

To clone the intended upstream region of *SCR2* of 1.29 kbp, PCR amplification was carried out on the genomic DNA of a salt tolerant rice land race *Mundan* using *Nipponbare* genomic sequence genome specific primers were used for amplification and cloning of upstream region: forward Primer: 5' – ATCTACTGTTGAAACCGC -3' and reverse primer: 5' – GTACCAGCGTGTCATTTG -3'. PCR was performed, for total reaction volume of 20 µL. The conditions opted for PCR amplification of promoter region was: Denaturation for 98 ºC (2 min), Annealing for 64 ºC (20 sec) and Extension for 72 ºC (2 min) for 30 cycles (Fig. 1). Proof reading Taq DNA polymerase (Primestar, Takara Bio Inc.) was used in PCR reaction. Given two other non-specific bands during the first round of PCR, the amplicon of the expected size alone was extracted and eluted from the gel using BioBasic kit, meant for the purpose of gel elution of the PCR products. The gel eluted PCR product was proceeded for cloning into pJET 1.2 plasmid vector.

Cloning of 1.29 kbp upstream of *SCR2* into pJET1.2 vector

The intermediary cloning vector, pJET1.2, it is a linearized plasmid capable of accommodating inserts of 6 bp to 10 kbp after recircularization of the double stranded plasmid DNA. The ligation reaction mixture of 20 µL contained, 10 µL of 2X reaction buffer, gel eluted PCR product of 1 µL (0.15 p mol), 1µL of DNA blunting enzyme, 1µL of pJET1.2 cloning vector (50 ng/µL), 1µL of T_4 DNA ligase, and 6µL nuclease free water. Further, ligation mixture was incubated at room temperature for 5 min.

Results and discussion

PCR amplification of upstream region of *SCR2* gene

PCR amplification revealed the presence of multiple bands of different sizes along with the expected amplicon of ~1.3 kbp (Fig. 1). The intended band of about 1.29 kbp was extracted from the gel and purified and cloned into pJET1.2 vector, an intermediary cloning vector. The recombinant pJET1.2 clone harbouring the 1.29 kbp upstream region of *SCR2* was verified by PCR (Fig. 2).

Identification of TSS and TATA-Box in the upstream sequence of SCR2

TSSPlant database was used to predict the transcription start site (TSS) in upstream region. TSS was identified at 1076 bp in the 1.29 kbp upstream sequence of *SCR2* (Table 1). The TSS and TATA-box positions are presented in figure 3. The upstream sequence comprised of 1076 bp region upstream of the predicted TSS and 214 bp included from the 5'-UTR of *SCR2*. The presence of a portion of the 5'- UTR has been included in some of the classical promoters maize ubiquitin promoter vector systems primarily to have an expressed expression of the transgene (Christenson *et al.,* 1996) [2].

In silico analysis of upstream sequence for the *cis*-regulatory elements

The upstream sequence of *SCR2* was subjected to motif analysis in order find the possible *cis*-acting motifs that regulate the spatio/temporal expression of the promoter. The *cis*-acting elements are regulatory sequences that are present on the promoters of genes and solely influence the expression of the gene. Scanning the upstream sequence of 1.29 kbp of *SCR2* gene from *Oryza sativa* cv. *Nipponbare* using PlantCARE database detected different *cis*-acting elements (Fig. 4).The, *in silico* analysis revealed the presence of 24 different *cis*-acting elements, in which *cis*-element CAAT-box (CAAT) was found to be present more in number (10) than any other *cis*-acting elements, followed by CCGTCC-box (3), A-box (3) (Table 2). CAAT-box motif is related to cellular development and have a role in meristem specific activation (Kaur *et al.,* 2017). The presence of the TGACG motif stimulates the formation of secondary metabolites by inhibiting or delaying the cell cycle at the G1/S checkpoint. The presence of GARE motif at 840 bp position, a gibberellins (GA) responsive element has been earlier implicated to control root meristem size (Achard *et al.,* 2009). GA signaling in the endodermis plays a key role in the regulation of whole root growth (Ubeda-Tomás *et al.,* 2008). Another role of GA signaling in the endodermis is promotion of cell divisions in the meristematic zone, which is required to enlarge the meristem zone during few days after germination. Achard *et al.* (2009) also reported the same conclusion as the role of GA signaling to control the root meristem size.

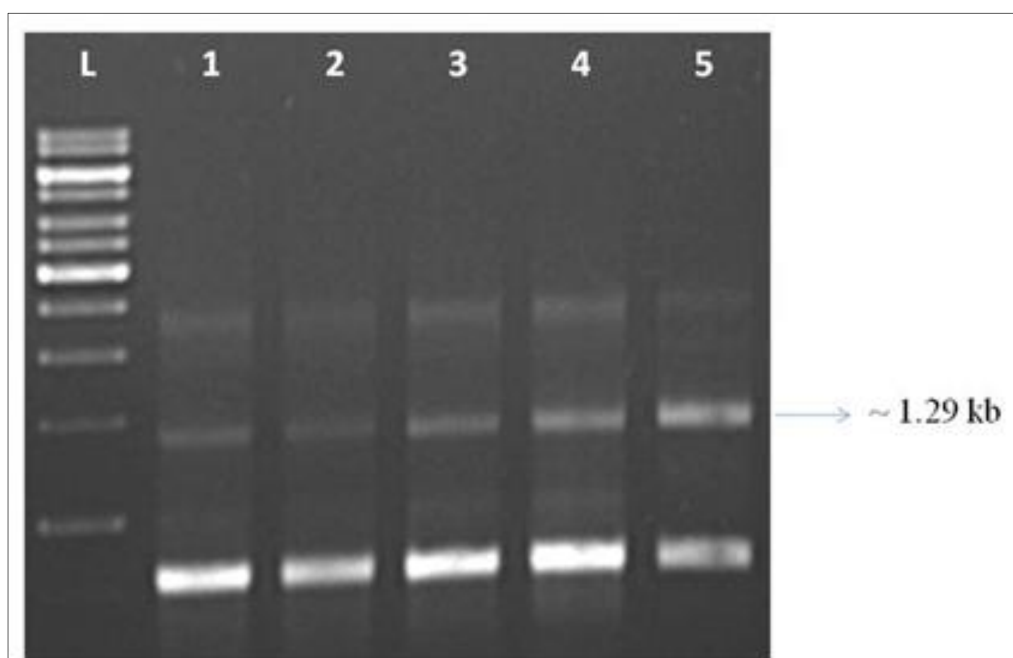
There are a few CAREs which are unique, CAREs such as A box and CCGTCC box are development-related motifs involved in the activation of meristem-specific expression. GARE and TATC box are regulatory elements involved in gibberellin responsiveness. This *in silico* analysis identified more *cis*-acting elements which are related to the development of root meristem.

Table 1: List of *cis*-regulatory elements predicted on the upstream sequence of *SCR2* using PLANTCARE database

TF motif	Sequence	Position	Abundance	Strand	Function
Unnamed__8	TCCACGTAGA	935	1	+	-
Unnamed__12	TCCACGTAGA	935	1	+	-
TATA-box	ATATAA	1042	1	+	core promoter element around -30 of transcription start
A-box	CCGTCC	225, 422, 468	3	+	cis-acting regulatory element
AAGAA-motif	GAAAGAA	601	1	+	
GCN4_motif	TGAGTCA	946	1	+	cis-regulatory element involved in endosperm expression
MYC	CATGTG	635	1	+	
GC-motif	CCCCCG	296	1	+	enhancer-like element involved in anoxic specific inducibility
CAT-box	GCCACT	74, 84	2	+	cis-acting regulatory element related to meristem expression
MYB recognition site	CCGTTG	106	1	+	-
AT-rich element	ATAGAAATCAA	1015	1	+	binding site of AT-rich DNA binding protein (ATBP-1)
CAAT-box	CAAT	571, 622, 707, 712, 748, 943, 996, 997, 1075, 1280	10	+	-
TGACG-motif	TGACG	1156	1	+	cis-acting regulatory element involved in the MeJA-responsiveness
Unnamed__4	CTCC	98, 927, 1069	3	+	-
MYB	CAACAG	1193, 1222	2	+	-
ABRE4	CACGTA	937	1	+	-
GARE-motif	TCTGTTG	840	1	+	gibberellin-responsive element
Myb-binding site	CAACAG	1193	1	+	-
WRE3	CCACCT	426	1	+	-
CCGTCC-box	CCGTCC	225, 422, 468	3	+	-
WUN-motif	TTATTACAT	1167	1	+	-
as-1	TGACG	1156	1	+	-
Unnamed__14	TCCACGTAGA	935, 225, 422	3	+	-
Unnamed__10	TCCACGTAGA	935	1	+	-

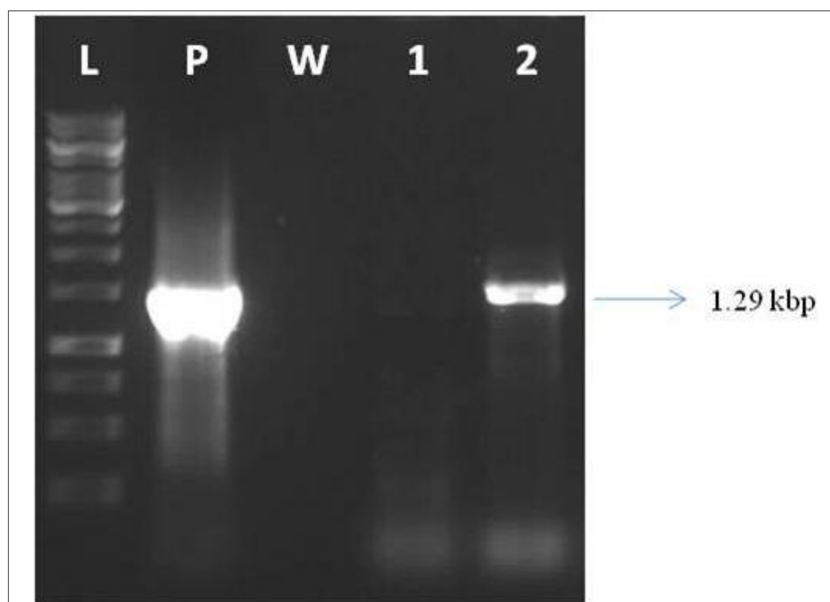
Table 2: Position of TATA box and transcription start site (TSS) on the 1.29 kbp upstream sequence of *SCR2* gene

Strand	TSS position	TSS score	TATA-Box position	TATA-Box Score
+	1076	1.9860	1042	7.5



Lanes: L- 1 kbp ladder; 1 – 5-Amplified products. The expected band corresponds to the arrow mark and other bands are non-specific in nature.

Fig 1: PCR amplification of *SCR2* upstream region of 1.29 kbp



Lanes: L -1 kbp ladder; P- Eluted PCR product; W-Water Control; 1- Non recombinant clone; 2- Recombinant clone

Fig 2: PCR of the recombinant clones of pJET1.2 harboring 1.29 kbp upstream region of *SCR2*

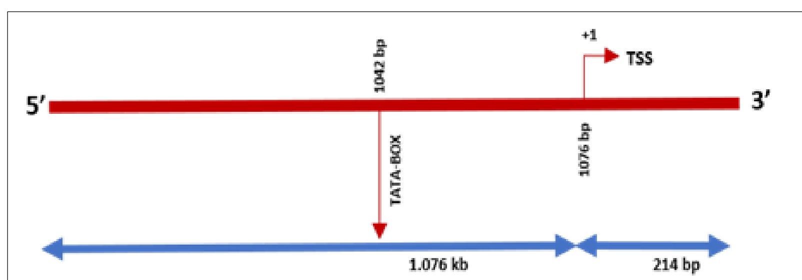


Fig 3: Architecture of the putative *SCR2* promoter depicting the predicted TATA box at 1042 bp and TSS region at 1076 bp and The + 1 site indicating the transcription start site of the putative *SCR2* promoter.

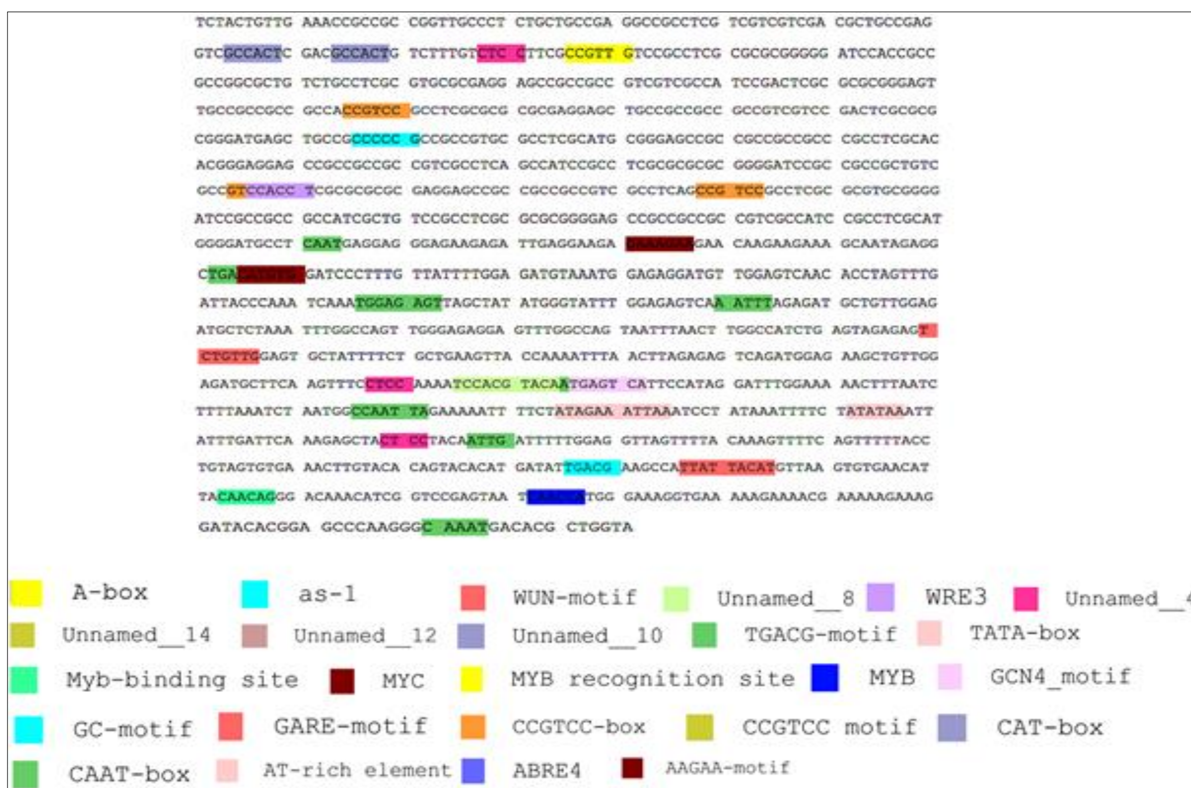


Fig 4: Identification of *cis*-regulatory elements present in 1.29 kbp upstream sequence of *SCR2* using plantcare

Conclusion

From earlier reports, SCARECROW has been bound to endodermis specific, In this investigation the upstream sequence of *SCR2* has been targeted for cloning and *in silico* anlaysis. *In silico* approach deduced the presence of several *cis*-elements in the 1.29 kbp upstream sequence of *SCR2* gene. This study sheds light on the relevance of a few selected *cis*-regulating elements on the upstream sequence of *SCR2* with respect to root development and endodermis differentiation. Further, the 1.29 kbp upstream region of *SCR2* gene was cloned in to an intermediary cloning vector pJET1.2. Furthering validation of the putative *SCR2* promoter will involve cloning of the *pSCR2* into a promoter cloner vector (pCAMBIA1305.1) and generation of stable rice transform ants and thereafter functional analysis to assess the endodermis specificity using GUS based reporter system. In future, such an endodermis specific promoter will pave way for selective expression of genes in the endodermis tissue facilitating precise regulation of the movement of solutes through the root apoplast with potential applications in developing crop plants for salt tolerance.

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