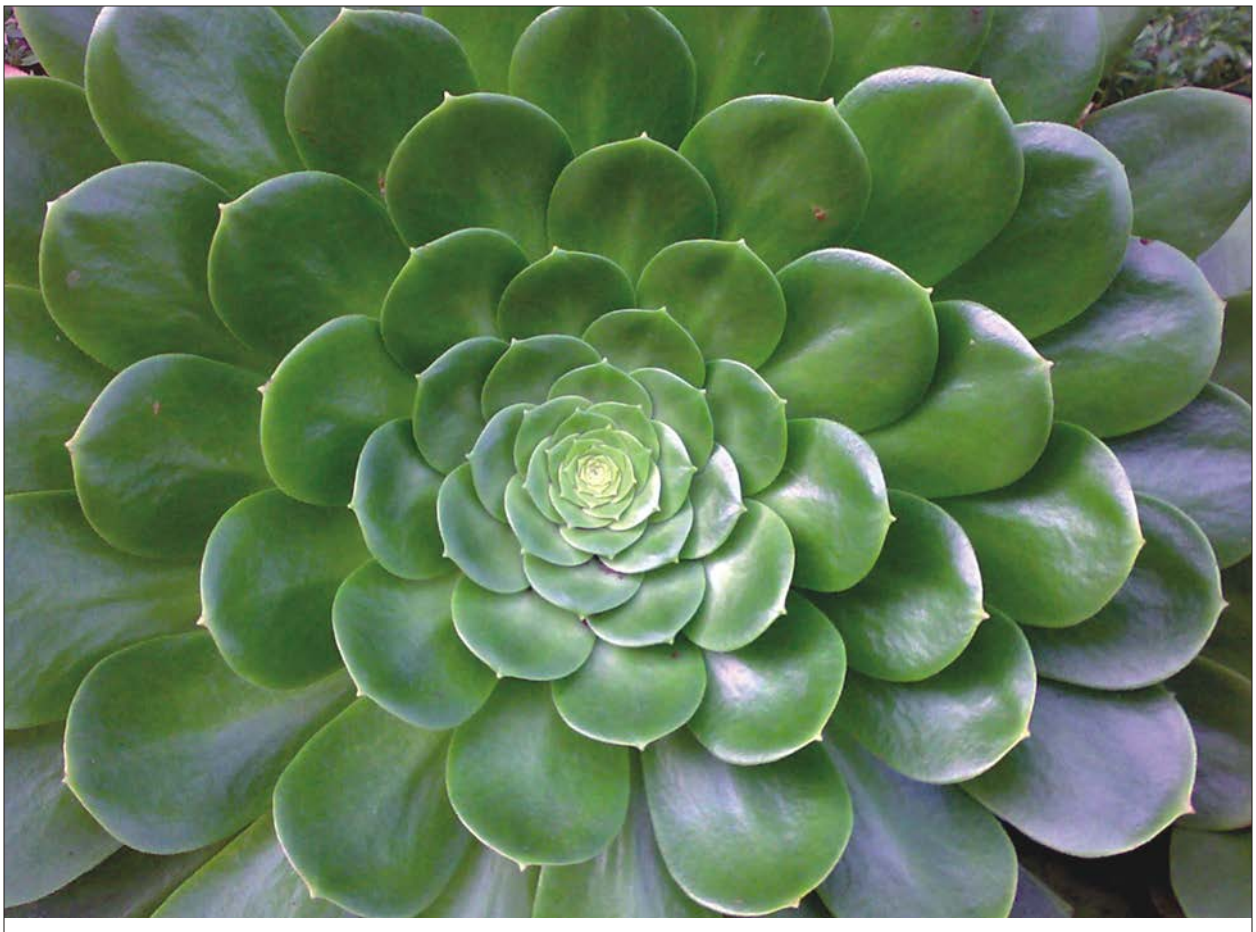

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Isolation, Sequencing and *in silico* Analysis of Sorghum (*Sorghum bicolor*) Sucrose Synthase Promoter

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Abstract: Regulation of expression of the transgene by use of appropriate promoters is most important for durability and specificity of resistance. Sucrose synthase promoter of cereals like Rice and maize was reported for their phloem specificity. Promoters are more efficient in their expression in the organism from which it has been isolated rather than in heterologous system. Taking these points into consideration, to control the sap sucking pests of Sorghum, Sucrose synthase promoter was isolated by Adapter PCR technique and *in silico* analysis of promoter sequence reveals the presence of phloem specific cis regulatory elements.

Key words: Sucrose synthase, adapter PCR, tissue specific promoter

INTRODUCTION

Cereals are the most important food crops among which sorghum is the fifth most important crop in the world. Sorghum is mainly cultivated in semi arid tropics and is extensively grown in Africa, Asia and North America. Stem borers cause major yield losses in cereals. Sorghum yield and yield stability are hampered by several insect species. The sorghum stem borer (*Chilo partellus*) is one of the serious pests of sorghum which infects the crop usually a month after sowing and causes dead hearts. Shoot fly is an important insect pest of seedling sorghum primarily in Asia and Africa. Depending on the plant stage during insect attack, feeding may lead to loss of leaf area, dead hearts, or stem and peduncle tunneling. Although each kind of damage may result in serious economic loss, not all are significant in a given location.

During development and differentiation, plants need to integrate a wide range of tissue, developmental and environmental signals to regulate complex patterns of gene expression (Karam and Singh, 1998). Manipulating the genetic makeup of plants to improve valuable traits such as seed oil composition, to make them insect pest resistant, pathogenic disease resistant, herbicide resistant or to introduce novel characteristics like production of vaccines or plastics is a challenging task. Appropriate control of genes that are introduced into plants is critical to success in this undertaking and the need for precision is dictated by the fundamental organization of plants as complex living systems (Datta and Selvaraj, 1995). For efficient pest control, it is important that effective levels of insecticidal proteins are expressed at the site where the insects feed. Regulation of expression of the transgene by use of appropriate promoters is most important for durability and specificity of resistance.

Sucrose synthase in source tissues is involved in phloem loading and phloem transport (Martin *et al*., 1993; Fu and Park, 1995; Hanggi and Fleming, 2001). Sucrose synthase genes have been isolated from many of plants, mostly from starch storing plants such as maize (Werr *et al*., 1985), rice (Wang *et al*., 1992; Yu *et al*., 1992), wheat (Marana *et al*., 1990), potato (Salanoubat and Belliard, 1987; Fu *et al*., 1995), pea (Barratt *et al*., 2001), bean (Silvente *et al*., 2003) as well as

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Arabidopsis thaliana (Chopra *et al*., 1992; Martin *et al*., 1993). Many plants have multiple, distinct isoforms of sucrose synthase genes, which have different patterns of expression in different organs of plant (Marana *et al*., 1990; Zeng *et al*., 1998; Barratt *et al*., 2001; Klotz *et al*., 2003; Kateri *et al*., 2006; Haagenson *et al*., 2006). For example, the two genes encoding sucrose synthase in maize Sh1 and Sus1 showed opposite responses to changes in tissue carbohydrate status (Koch *et al*., 1992).

Rice (Shi *et al*., 1994) and maize (Yang and Russell, 1990) sucrose synthase gene promoters have been reported for phloem specific expression. The present study to isolate Sucrose synthase gene promoter from sorghum by adapter PCR technique with the objective of expressing insecticidal genes in a tissue specific manner under the control of homologous promoter to control pests which targets phloem.

MATERIALS AND METHODS

Isolation of Plant DNA

Sorghum seedlings were grown in tissue culture lab of National Research Centre on Plant Biotechnology, New Delhi during June, 2003. Two weeks old sorghum seedlings grown in ½ MS medium (Murashige and Skoog, 1962) were taken for total genomic DNA isolation. Sorghum (var. CSV 15) seeds were kept in 0.1% $HgCl_2$ for 10 min with intermittent shaking. After decanting 0.1% $HgCl_2$ solution, the sorghum seeds were thoroughly washed in sterile distilled water two times and then placed in tissue culture bottle containing ½ MS medium. Sorghum seeds were kept at 28°C (BOD incubator) for germination. Sorghum genomic DNA was isolated by CTAB method (Dellaporta *et al*., 1983).

PCR Amplification of Sorghum Sucrose Synthase Gene Fragment

Multiple sequence alignment of sucrose synthase gene sequences of various cereals like rice, maize and barley were accessed from NCBI Genbank database and multiple sequence alignment was done using MegAlign programme of DNA star software. Primer sequences were designed at the conserved coding region to amplify a sucrose synthase gene fragment from sorghum. Gp1F 5' CTCCTCTCATCCCAATG 3' forward primer was designed at second exon position two and Gp2R 5' CCCCTTCTCCAAACCAAG 3' reverse primer was designed at sixth exon position. Sorghum genomic DNA was used as template. 1.5 kb amplicon obtained was purified using Qiaquick PCR purification kit (QIAGEN, Germany). UA cloning vector (QIAGEN PCR Cloning Kit) was used to clone the PCR amplified product. The recombinant clone was sequenced using T_7 and SP_6 reverse primers. Sequencing was carried out by Sanger dideoxy DNA sequencing method.

Southern Hybridization

Localization of particular sequences within genomic DNA is usually accomplished by the transfer techniques described by Southern (1975).

DNA Restriction and Electrophoresis

Ten to fifteen microgram of plant DNA was taken and restricted by restriction enzymes such as *BamHI, EcoRI, BglII* and *SalI* separately. Five unit of each enzyme were taken per μg of DNA in 1X reaction buffer and incubated overnight at 37°C. After incubation the reaction was stopped by addition of 1 mM EDTA. The digested DNA fragments were resolved by gel electrophoresis. For separation of DNA, 0.8% agarose gel was prepared in 1X TAE. The voltage applied across the gel was 40 V for 8-10 h. *HindIII* digested DNA was used as marker. Ethidium bromide at the rate of 0.5 μg mL^{-1} was added to the gel before casting the gel.

Transfer to Membranes

After the completion of run, the position of marker bands was recorded on a cellophane sheet and the gel was soaked in 0.25 N HCl for 10-15 min with gentle shaking for depurination. The gel was then washed twice with double distilled sterile water and 0.4 N NaOH for 45 min to 1 h. The gel was carefully placed on a Whatman No. 3 blotting paper whole sides dip in a tank of solution containing 0.4 N NaOH and 1.5 M NaCl over a clean glass plate. A nylon N^+ membrane (Hybond-N^+, Amersham) was cut to same size as the gel and placed over the gel after soaking it in 0.4 N NaOH for 30 sec.

Three to five pieces of Whatman No. 3 blotting paper were cut to same size as the gel, soaked in 0.4 N NaOH and placed on the membrane. Air bubbled trapped between the gel and membrane was removed by rotating a clean glass rod over the Whatman paper. Then 5-7 cm thick stack of blotting sheet (of the same size as the gel), a glass plate and weight of approximately 500 g were placed over it. The capillary transfer was allowed continue for 14 to 15 h, after which the assembly was dismantled. The membrane was cut at the side of *HindIII* digested marker loaded position and the membrane was cross-linked over UV transilluminator for 30 sec, air-dried and kept in a desiccator till further use.

Pre-Hybridization

The membrane was placed in a clean dry hybridization bottle (Amersham) and 10-20 mL of pre-hybridization buffer was allowed to continue for 2-4 h at 65°C at a speed of 10-15 rpm in a hybridization oven.

Preparation of Probe

Deca Label™ DNA labeling kit (MBI Fermentas) was used for the preparation of probe.

Washing of Filter and Autoradiography

High stringency i.e., low buffer and high temperature was employed during washing of filters. After decanting the hybridization solutions to safe disposal, the filter was washed with 2X SSPE, 0.1% w/v SDS at 55°C for 10 min and subsequent wash with 0.5X SSPE and 0.2X SSPE with 0.1% w/v SDS. The washed membrane was then kept on a support (usual X-ray film) covered with saran wrap after removing trapped air bubbles, if any and then exposed to X-ray film (Hyperfilm, Amersham) in a cassette with an intensifying screen.

The cassette was kept at -70°C for appropriate exposure time based on the CPM of ^{32}P on the membrane and the stringency of washing the film was developed in Kodak developer for 2-3 min, rinsed in distilled water and fixed in Kodak fixer for 10 min. The film was washed thoroughly under running tap water and air-dried.

Adapter PCR

Adapter PCR technique was followed to get the upstream sequence of sorghum sucrose synthase gene. It is kind of PCR technique in which one gene specific primer and one random primer is used. Adapter PCR technique was done to get the upstream sequence of sorghum sucrose synthase gene.

Restriction Digestion of Genomic DNA

Sorghum genomic DNA was taken (15-20 μg) and digested overnight with the *BamHI*, restriction enzyme. This enzyme will produce the same compatible overhang and finally the digested DNA was purified using PCR purification kit (QIAGEN).

End Filling Reaction

The digested DNA was partially end filled with dGTP and dATP (2 mM each) in the presence of 10x klenow buffer and klenow enzyme (10 U μL^{-1}) in a reaction volume of 50 μL and was incubated at 37°C for 30 min followed by purification using PCR purification kit (QIAGEN).

Adapter Preparation

Two partially complementary primers 33 and 11 mer in a equimolar concentration was taken and kept at 95°C for 3 min and allowed to come down gradually to the room temperature for the purpose of annealing.

Ligation with Adapters

The partially end filled DNA was ligated to the adapter in a ligation reaction at 16°C for 24 h in a 20 μL reaction volume containing 8-12 mg DNA, 1.5 μg adapter and 1 μL of T_4 DNA ligase (2 U μL^{-1}) excess adapters were removed again through Qiaquick columns (QIAGEN).

PCR Amplification

The adapter ligated genomic DNA was suitably diluted and used for amplification in a 50 μL reaction mixture under the following amplification conditions.

• 94°C	3 min	Initial denaturation
• 92°C	1 min	
• 55°C	1 min	
• 72°C	2 min	Step 2-4 cycled for 30 times
• 72°C	5 min	Final extension

T_7 and gene specific primers were used for amplification. Genomic DNA amplified with T_7 primer alone was used as negative control. The purified products of first round of amplification were re-amplified with the second gene-specific primer.

Cloning and Sequencing

The DNA bands obtained after re-amplification were cloned in P drive TA cloning vector. The recombinant clone was sequenced using T_7 and SP_6 reverse primers. Sequencing was carried out by Sanger dideoxy DNA sequencing method.

Adapter PCR (Flow Chart)

- Adapter primers
  - 5' GTA ATA CGA CTC ACT ATA GGG CTT TGC GGC CGC 3'
  - 3' *ACG CCG GCG CT 5'

↓

To get the adapter, equimolar concentration of adapter primers 1 and 2 was kept at 95°C for 3 min.

↓

5' GTA ATA CGA CTC ACT ATA GGG CTT TGC GGC CGC 3'
3' *ACG CCG GCG CT 5'

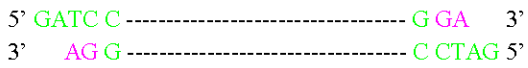
- Sorghum genomic DNA restriction with *BamHI*

↓ ↓

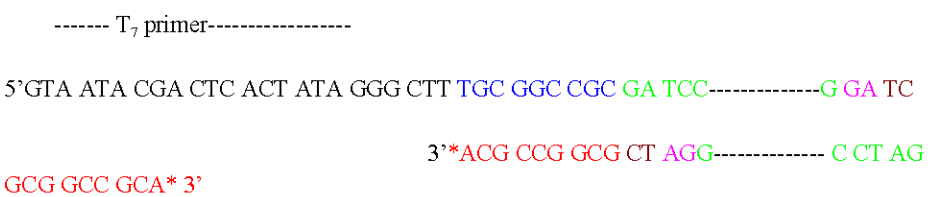
5' G GATC C ---------- G GATC C 3'
3' C CTAG G ---------- C CTAG G 5'

↑ ↑

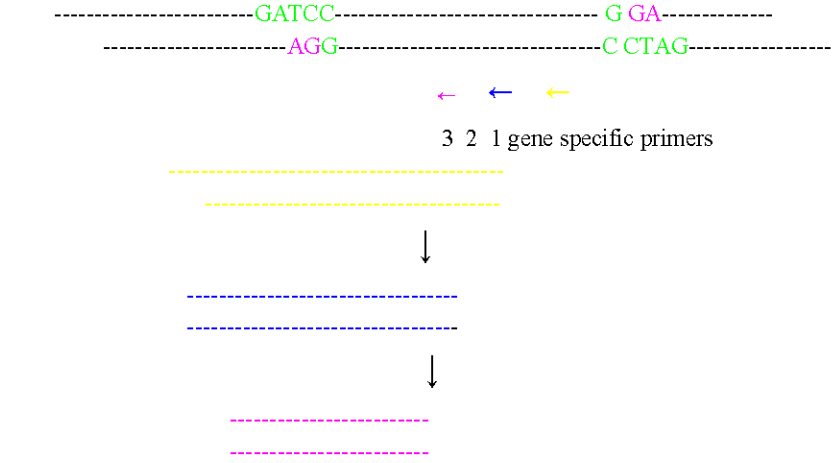
- Gel extraction and elution of 6 and 4 kb DNA fragment that were obtained as hybridization signals when *BamHI* restricted sorghum genomic DNA was southern hybridized.
- End filling with dATP and dGTP



- Ligation of the adapter to *BamHI* restricted and filled genomic DNA



- PCR amplification with T_7 primer and gene specific primers



Cloning and Sequencing

The DNA band obtained in the third round PCR with Gp5R gene specific primer was cloned in pDrive TA cloning vector. The recombinant clone was sequenced using T_7 and SP_6 reverse primers. Sequencing was carried out by Sanger's dideoxy DNA sequencing method.

RESULTS

Primer Design and PCR Amplification

Multiple sequence alignment of sucrose synthase gene sequence from different cereals revealed that it is highly conserved at their coding regions. Highly conserved exon positions were chosen to

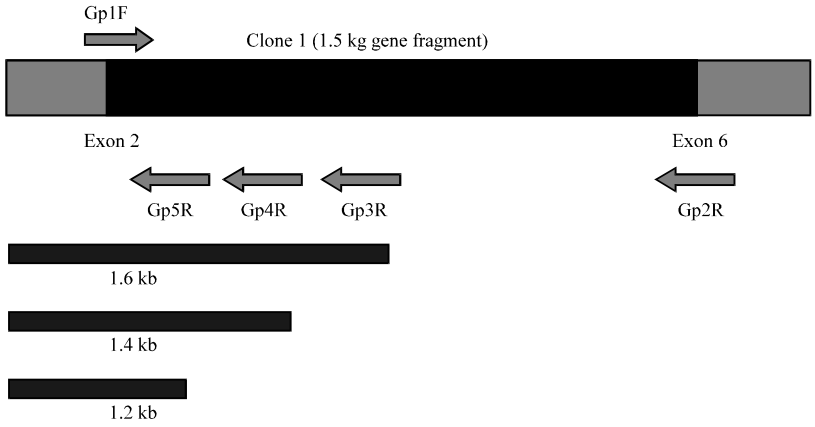


Fig. 1: Schematic diagram showing the exon position where primers were designed. Gp: Gene specific primer, F: Forward primer, R: Reverse primer

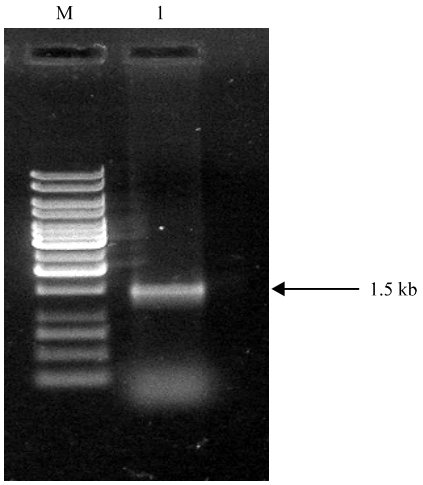


Fig. 2: PCR amplification of Sorghum sucrose synthase gene fragment using second set of gene specific primers. Lane M: 1 kb Ladder and Lane 1: PCR product of Sorghum sucrose synthase gene fragment II

design primers and using those to amplify sucrose synthase gene fragment form sorghum. Schematic diagram showing the exon position where the primers were designed (Fig. 1) and the DNA fragment size of 1.5 kb was amplified and cloned in UA cloning vector is shown in Fig. 2 and 3. The amplicon was sequenced and multiple sequence alignment was done with *Rss1*, *Rss2* and *Rss3*, of rice, SUC 2, SUCI of maize, Ss_2 of *Hordeum vulgare* and *Saccharum officinarum*. The amplicon shared homology at the exon position second and six as expected and confirmed as Sorghum sucrose synthase gene fragment (Fig. 4).

Southern Hybridization of Sorghum Genomic DNA

Genomic DNA was isolated from two weeks old sorghum seedlings and RNase treatment was given to make the genomic DNA free of RNA. Southern hybridization was carried out to find out

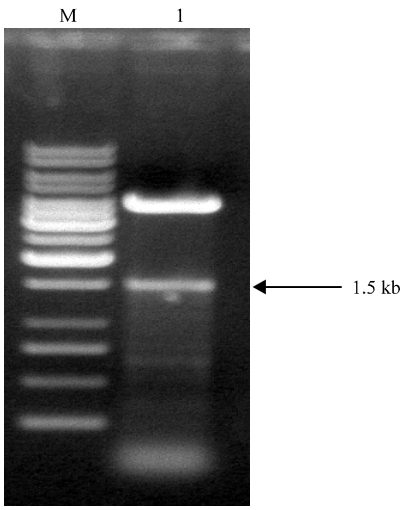


Fig. 3: Sorghum sucrose synthase gene fragment II of ~ 1.5 kb size. Lane M: 1 kb Ladder and Lane 1: P drive UA cloning vector having sorghum sucrose synthase gene fragment II restricted with *EcoRI*

1 CTCCTCTCAT CCCAATGAGC TGATTGCACT CTTCCTCCAG GTGGGCATAC CAAAATATGT
61 AACTTGCATT TCATTTCCTG TACTGGAATT TGTTAATTTG GTATTCTCTT CATCCCAAAT
121 GTAAACACGA GCATATGCAA CTTCTTTCTT GGTTTCTTTT GTTAACACCA TCATGCATGC
181 TAATTGCTAT TCATCATCGA CTCATTGATC ATATATAATG ATTTTATGAT AGGAGATTA
241 TTGATTGTAA AGCATAGTGT TGCTGCTCTT CAGTTTTTGA AGCCTTTTGG TTTGATTAGT
301 ACAATTAGTT GATAAGACAG TATACTTTGT GGTACATCAT TTGGCAGATTGTTTGACTTT
361 AGTTGGTACA GTGCCATTTA ATATTTACAT CCTTCAGATC TAAATAGGAT TAAAATGTC
421 CATCACAGCA GGGGAAAAGG TACATGATAT GAGATGTAAC ATCCATTTTA TGTGAATA
481 TCACTTTTAC AGGTATGTTA ACCAGGGCAA GGGAATGCTT CAGCGCCATC ACTGCTTGC
541 TGAGTTTGAT GCCCTGTTTG ATAGTGACAA GGAGAAGTAT GCGCCC? (Gap of 250-300 bp)
? TTCG AAGACTTTCT
601 TCGTGCTGCT CAGTAACACT GCTGAGATGC CTGCTTGAGT GATTGCGCCA TCAACAGCA
661 ACTTGTGCTG AGCTGATTTG AGCCTTCAAT GCTCGTTCCC TCGTCCTTCC ATGTCAAAGT
721 CCATGGGAAT GGAGTGCAAT TCTTAACCGA CACCTGTCTT CCAAGTTGTT CAGGACAAG
781 GAGAGCCTGT ACCCATTGCT GAATTTCCTC AAAGCCCATA CTACAAGGGCACGGTGAGC
841 TTACAATTCA GAATCTTCCA AGCACATGCT TCACAATGGA TGATGACAAT TTTATTTAG
901 GAACTTTACA TAATCTGAAA ATGGATTAAA TGATGCCACC CAACTCCCTC TTTGTAAGT
961 CTTTTTTTTT TCTGTTACAG ACGATGATGT TGAATGACAG AATTCAGAGCCTCCGTGGGC
1021 TCCAGTCATC CCTTAGAAAG GCAGAAGAGT ATCTACTGAGGTCCCTCAAGACACTCCCT
1081 ACTCAGAGTT CAACCATAGG TGATTCATCA ATAAATTGTC CTTGCCATTT ACTTTGGTT
1141 GAACTAGCAA TGTATTAACTGCTTGTATGCCACCATGATCTGCATTAGGTTCCAAGAGC
1201 TTCCAAGAGC TTGGTTTGGA GAAGGGG

Fig. 4: Nucleotide sequence of sorghum sucrose synthase gene fragment II Gp1F-Gp2R. Note: Highlighted portions are exon sequences sharing homology with cereal sucrose synthases

suitable restriction enzyme and design adapter with complementary overhang. Sorghum genomic DNA was restricted independently with various kinds of enzymes such as *BamHI*, *EcoRI*, *BglII* and *PstI*. 1.5 kb Sorghum sucrose synthase gene fragment obtained by PCR amplification using primers Gp1F and Gp2R was used as hybridization probe. Lane loaded with *BamHI* restriction enzyme digested genomic DNA gave two hybridization signals of sizes about 6 and 4 kb. *EcoRI* digested sample gave a hybridization signal size of around 10 kb. *PstI* and *BglII* digested sample gave multiple hybridization signals of various size ranges (Fig. 5).

Table 1: Nucleotide sequences of primers

Primers	Sequence
Gp1F	5' CTCCTCTCATCCCAATG 3'
Gp2R	5' CCCCTTCTCCAAACCAAG 3'
Gp3R	5' CCTGCTGTGATGGACAT 3'
Gp4R	5' GATCAATGAGTCGATGA 3'
Gp5R	5' CATTGGGATGAGAGGAG 3'
Ap1	5' GTA ATA CGA CTC ACT ATA GGG CTT TGC GGC CGC 3'
Ap2	5' TCG CGG CCG CA 3'
Ap3	5'GTA ATA CGA CTC ACT ATA GGG C 3'

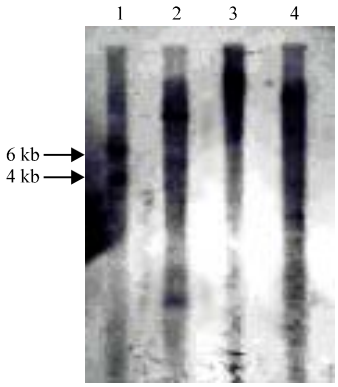


Fig. 5: Southern hybridization of sorghum genomic DNA using sucrose synthase gene fragment as probe. Lane 1: Sorghum genomic DNA restricted with *BamHI* restriction enzyme showing two hybridization signals with the approximate size band of 6 and 4 kb, Lane 2: Sorghum genomic DNA restricted with *EcoRI* restriction enzyme, Lane 3: Sorghum genomic DNA restricted with *BglII* restriction enzyme and Lane 4: Sorghum genomic DNA restricted with *PstI* restriction enzyme

Adapter PCR

Adapter PCR was carried out to amplify the upstream region of sorghum sucrose synthase gene. Three nested sets of primers with the interval of 200 bp were designed near the second exon position (Fig. 1, Table 1). In the Southern hybridization, *BamHI* restricted sorghum genomic DNA sample gave two hybridization signals of size 4 and 6 kb. To carry out adapter PCR, Sorghum genomic DNA of same concentration (5-7 μg which was taken for doing Southern hybridization) was taken and over nightly restricted with *BamHI*, restriction enzyme and agarose gel electrophoresis was carried out at low voltage. In the UV illuminator, the restricted genomic DNA was observed as smear. The smear corresponding to ~6 and 4 kb marker band was cut and gel purified. Both 6 and 4 kb fragments which gave hybridization signals with *BamHI* restriction enzymes were taken for Adapter ligation as any one of fragment might carry the upstream sequence. Adapter primers were designed to include T_7 promoter primer sequence. Adapter primers were end filled with d ATP and d GTP using klenow enzyme in order to have a complementary cohesive sequence, which can be ligated to *BamHI*, restricted fragment. Adapter primers were ligated to gel purified *BamHI* digested 4 and 6 kb fragments independently. PCR was carried out by T_7 as forward primer and gene specific primer as reverse primer using adapter ligated to 4 and 6 kb fragment as template. To avoid non-specific amplification, three nested sets of gene specific primers were designed. First PCR was carried out with T_7 primer and extreme outward gene specific primer (Gp3R). The size of the DNA band obtained was ~1.6 kb. The PCR product obtained in the first set of reaction was used as template in the second PCR reaction where forward

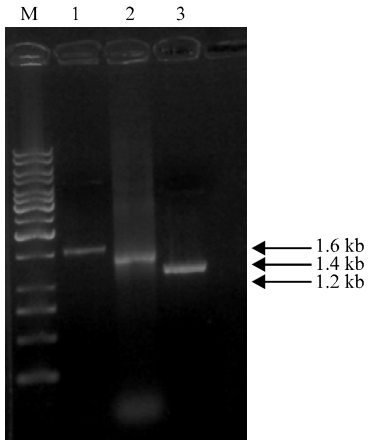


Fig. 6: Adapter PCR amplification of upstream sequence of Sorghum. Sucrose synthase gene using nested set of gene specific primers. Lane M: 1 kb Ladder, Lane 1: ~ 1.6 kb PCR product obtained using gene specific primer (Gp3R) and T_7 Primer, Lane: 2 ~1.4 kb PCR product obtained using gene specific primer (Gp4R) and T_7 Primer and Lane: 3 ~1.2 kb PCR product obtained using gene specific primer (Gp5R) and T_7 Primer

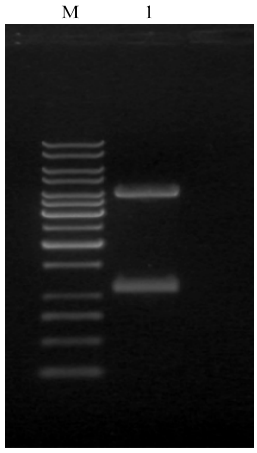


Fig. 7: Sorghum sucrose synthase promoter cloned in P drive UA cloning vector. Lane M: 1 kb Ladder and Lane 1: Pdrive UA cloning vector having sorghum sucrose synthase promoter of ~1.2 kb size restricted with *EcoRI*

and reverse primers were T_7 and 2nd outward gene specific primer GP4R, respectively. In the second PCR 1.4 kb DNA band was obtained when the reaction sample was electrophoresed at 0.8% agarose gel. In 3rd PCR reaction, 2nd PCR reaction product was used as template. The forward and reverse primers were T_7 primer and Gp5R, respectively. The size of PCR product was 1.2 kb (Fig. 6). The amplicon size of ~1.2 kb was cloned in UA cloning vector (Fig. 7) and sequencing was carried out by Sanger dideoxy DNA sequencing method (Fig. 8).

***In silico* Analysis of Promoter**

Plant CARE is a database of plant cis-acting regulatory elements for *in silico* analysis of promoter sequences. The 1.2 kb sorghum sucrose synthase promoter sequence was analysed in Plant CARE

Table 2: Sorghum sucrose synthase promoter sharing homology with cis-regulatory elements of other Cereals (Ref: Plant CARE software)

Variables	Organism	Position	Strand	Sequence	Functions
A Box	*O. sativa*	176	+	TATCtatcatt	-
	-	654	+	TATCaaccgac	
CAAT Box	*H. vulgare*	439	+	GCCAAT	-
	H. vulgare	795	+	CAAT	
	Z. mays	881	+	CCAAt	
GC Motif	*Z. mays*	424	+	CcCCCgt	Enhancer like element involved in endosperm expression
	O. sativa	636	+	atCGCGCa	
	Z. mays	851	-	gcCCCG	
GCN 4 Motif	*O. sativa*	115	-	ACTGAGC	Cis-regulatory element involved in endosperm expression
	O. sativa	442	+	tggGTCA	
	O. sativa	568	+	taaGTCA	
-	*O. sativa*	541	+	TACAaac	Core promoter element around -30 of transcription start
AS 2 Box	*N. tabacum*	284	-	GTAGTAATAG	Involved in shoot specific and light responsiveness
P Box	*O. sativa*	138	+	CCTTttt	Gibberellin responsive element
	O. sativa	449	+	CCTTtgg	
I Box	*T. aestivum*	718	+	AGATaggg	Part of a light responsive element
	T. aestivum	381	-	AGATagga	
	Z. mays	248	-	tGATAtggtt	
G Box	*Z. mays*	170	+	CATGtt	Cis-acting regulatory element involved in light responsiveness
	Z. mays	593	+	CACGat	
	H. vulgare	642	+	CaGGTG	
	H. vulgare	14	+	gttACGTAtc	
ABRE					Cis-element involved in the abscisic acid responsiveness

1 CATGCTGCAG ACGCGTTACG TATCGGATCC AGAATTCGTG ATTCGAGCTC
51 GCAATCAGCT CATTGGCCAG TACAGGAAAT GAAATGCAAG GCGAAGGTAT
101 AAAAGTAGAG CTGGTTGACT CGACATTTTT TCCAAAAGCC TTTTTTAAGA
151 CTAACACAAT GTAAGAGCAT CATGTTTATC TATCATTAGC CTACCACTAG
201 AATAGCACCT GTACTACAAC ACATCACTTG ATTATTACAA GCAATAATAA
251 CCATATCAAG TTCATCATAT GTTCTTCTTG CTATCATCAT TATCTTGATC
301 CAAGTTCATT AGTGGATGCT ACGTTTGCCG CTGCTCTGTC AAGTTCTCAC
351 TGACCGGGAG AGATGGCGAT TCGAATCGAA TTCCTATCTA GCTGGAGGGT
401 TATTCCTAGC ACTCACCCAA GCATCCCCCG TCGGAGAGCC AATGGGTCAC
451 CTTTGGTACG ACTCAGGAAT CGCGACTCCG ATCAGCGCCG CACTCCCAGG
501 GCTACCACTC TGCCAGGGAG GTCAGGAATT TTAACTCACA CTACAAACTT
551 GCAAAACTCT GCCCGGCTTA AGTCAGATTA ATCAGGTTCC ATTCACGATA
601 GCACATATTG ACCATCGTGA TCCGACATAA CCTTATATCG CGCAGGTGAC
651 AGGATATCAA CCGACTTCTA CCGATTAAAG CATGGCTAAG CTGCTACTCG
701 ATCCTAACTC TATAACCAAG ATAGGGTAAA GTATCTGGAC AAGGATTGAG
751 TAAAATGCAG CAAATGTTTC GTCACAACTC CTATACTTAA TGCAACAATA
801 GATATAACAT ATTAAATAAA CTTTCAAAAG TAAAGGGGGA CTTAGAATGC
851 TCCGGGGCTT GCATTTCATT TCCTGTACTG CCCAATGAGC TGATTGCGAG
901 CTCGCTGATC GGATTTGCAA GCTGGTATCA CGAGTGGCAG CTCGACCAAA
951 CCTCCTGATT ATTCAGCCGC CGGATTTTTC CTGAGGGCTA ATATCAAAGG
1001 TTGAAATATG CGGTCTCCGA GAAGGACTGG TGGTCGTATG TGCGTTAGCC
1051 GTGCTAAGCT TTCCCTTCCG TTTCGGTTTC GCGCAGGCGA GACTCCGGCG
1101 CCGGTTTATA TTGCCTGGCT TTGACATGAA ACGCGCCTTA TCTCCTCTCA
1151 TCCCAATG

Fig. 8: Nucleotide sequence of sucrose synthase promoter of sorghum

database. The conserved box and the cis-elements which were sharing similarity with other plant promoter cis-elements were given in Table 2. The transcription start site was found to be at 578 bp. TATA box is reported to be important for RNA polymerase II recognition. There are TATA less

promoters, which were reported to be found in nuclear encoded photosynthetic genes. The consensus sequence for TATA box was TACAaac found at 541th nucleotide position. This sequence is homologous to the consensus sequence for TATA box for rice. The CAAT box was found upstream of TATA box at nucleotide position 439. This CAAT box was found to be homologous with CAAT box found in Barley. This is a common cis-acting element in promoter and enhancer region. A-box motif, cis-acting element conserved in alpha-amylase promoters was found at the nucleotide position 176 bp. The GC-motif with the consensus sequence at CGCGCa was found at the nucleotide position 636 down stream of TATA box, which shared homology with rice in which it is said to be enhancer like element involved in endosperm expression. Similarly GCN4 motif was found at 442 bp, which is 99 bp upstream of TATA box, which is a cis- regulatory element, involved in endosperm expression. I box was found at two positions 248 and 381 bp and G box was found at 170 and 593 bp. Both are cis-acting element involved in light responsiveness. P box is gibberellin responsive element (ABRE) a cis acting element involved in the abcisic acid responsiveness was found at nucleotide position 317 bp. Two cis acting elements *BoxII* at nucleotide position 600 and GATA motif at three positions 250, 278 and 381 bp in the negative strand were found. It was reported that both act combinatorily in phloem specific expression of RTBV promoter (Yin *et al*., 1997). As2 box was found in the negative strand at position 284 bp responsible for shoot specific expression.

DISCUSSION

Sucrose synthase is found in all plant tissues and is found at high levels particularly in sink tissues. In monocotyledonous plants sucrose synthase is encoded by a small gene family. Phloem specific expression of sucrose synthase gene promoters have been reported in monocots such as *Rss*1 of Rice (Shi *et al*., 1994) and Sh1 of maize (Yang and Russell, 1990) and dicots such as Sus3 of Potato (Fu *et al*., 1995) and Asus1 of Arabidopsis (Martin *et al*., 1993).

Southern hybridization of Sorghum genomic DNA restricted with different restriction enzymes such as, *EcoRI*, *BglII* and *PstI* was done using Sorghum Sucrose synthase gene fragment as a probe. There were two hybridization signals corresponding to the size of 6 and 4 kb found with restricted DNA sample (Lane 1). Lane 2 loaded with *EcoRI* digested DNA sample (Fig. 5) was observed with three bands of size 8, 5 and 1 kb. Lane 3 with *BglII* digested DNA sample was observed with two hybridization signals above 10 kb and around 7 kb. Lane 4 with *PstI* restricted DNA sample was with four hybridization signals of different size i.e., 9, 7, 4 and 3 kb. Sorghum genomic DNA restricted with four different restriction enzymes (*BamHI*, *EcoRI*, *BglII* and *PstI*) all four of them was observed with minimum of two hybridization signals. This suggests that there should be at least two sucrose synthase isoforms independently coded by two genes in Sorghum. Lane 2 and Lane 4 was observed more than two hybridization signals indicates the possibility of presence of two restriction sites within the gene makes them get detected twice.

Sucrose synthase promoter of sorghum was amplified by Adapter PCR technique, which shared sequence conservation with neither *Rss1* promoter nor with Maize Sh1 promoter. But it has similar cis regulatory elements as that of Maize Sh1 and Rice *Rss1* promoter. It has cis elements for phloem specific expression such as Box II and GATA motif as that of RTBV promoter. RTBV promoter has three cis elements viz., Box II, the ASL Box and the GATA motif, which were shown to confer phloem specific gene expression (Chen *et al*., 1997).

The GATA family of transcriptional factors is zinc finger proteins that specifically recognize the GATA consensus sequences and are proven to be important for tissue specific gene expression, differentiation and development in mammalian systems (Simon, 1995). GATA motif has been found in most of the light regulated promoters and has been shown to be important for the activity of these promoters (Terzaghi and Cashmore, 1995). A GATA motif found in 17 bp element involved in binding of the protein factor Gs3A-F1 to the minimal-132 Gs3A gene promoter, which retained phloem

specific gene expression (Brears *et al*., 1991). Likewise, the phloem specific AHA3 promoter has a GATA motif, although its function has not been characterized, a similar GATA motif was found at the nucleotide position 250 and 278 of sorghum sucrose synthase promoter which suggests that it may be involved in phloem-specific expression.

Sucrose synthesis plays an important role in seed development. GCN4 motif was found at the nucleotide position 442 and 568, which shares sequence similarity with rice GCN4 motif that is involved in endosperm expression. As2 box which was found at the nucleotide position 284 of minus stand, shares sequence similarity with N. tabacum, which is responsible for shoot specific expression.

Combinatorial interactions in transcriptional regulation have been well documented in plants (Benfey and Chua, 1990). For example, the combinatorial interactions of different tissue specific sub domains (Cis elements) results in constitutive expression from the CaMV 35S promoter (Benfey and Chua, 1990). Regulatory mechanism may reflect the evolution and differentiation of phloem and xylem. It has been proposed that phloem appeared much earlier during evolution of higher plants than did xylem and phloem always differentiates before xylem during plants development (Aloni, 1987). So, different combinations of different cis elements seem to serve as a code to direct spatial and temporal expression of different genes. To further understand the nature of these combinatorial interactions and their regulation by environmental signals, it may well be necessary to clone and characterize the factors binding to these elements.

The constitutive expression of the insecticidal gene in transgenic plants is likely to increase the risk of pests developing resistance and may also result in yield penalties as the plant directs more resources than necessary to the defense. To avoid this, tissue specific promoter can be used, thereby limiting exposure to the toxin, which could be expressed in those parts of the plant affected by insect. To manage the insects belonging to the suborder Homoptera, such as plant hoppers and aphids, which feed by sucking phloem sap of the transgenic plant, an insecticidal gene can be expressed in phloem tissues to achieve better results. The sorghum sucrose synthase promoter isolated in the present study can be used to direct phloem specific expression of such insecticidal genes.

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