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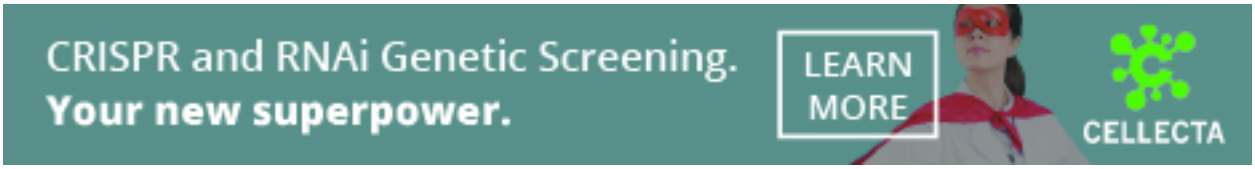

Amplification and Cloning of Sugarcane Sucrose Synthase cDNA by Anchored PCR

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We have used a strategy based on the polymerase chain reaction (PCR) to amplify and construct full-length sucrose synthase (SS) cDNA of sugarcane. Two SS-specific internal primers were synthesized based on their complementarity to published consensus sequences of the SS gene of maize and wheat. Amplification of full-length cDNA was achieved by an anchored PCR method utilizing primers which extend to 5′ and 3′ ends of specific cDNA. In the first step, a homopolymeric oligo(dC) tail was added to the 3′ end of single-stranded cDNAs. The two SS cDNAs were amplified, one with a 5′ end (SSp1) and the other with a 3′ end (SSp2) using one internal SS primer and the other anchored end primer. Finally, overlapping fragments were identified by restriction mapping, and the non-overlapping fragments were excised and religated to reconstruct full-length cDNA. Partial sequences of the reconstructed cDNAs (SS-5′ and SS-3′) were compared with the published SS sequences to confirm that the amplified DNA was a copy of the SS transcript.

The cultivated species of *Saccharum* accumulate high concentrations of sucrose, exceeding 700 mM, in stalk parenchyma tissue. Sucrose synthase (SS), sucrose phosphate synthase (SPS), and invertase was found to have significant roles in sucrose metabolism of sugarcane.[1–3] However, the precise role of each of these enzymes in synthesis, transport, and partitioning of sucrose is not clear. Cloning and characterization of these enzyme-encoding genes might help elucidate the function of these enzymes by molecular techniques such as in situ hybridization and antisense gene technology. In general, isolation of a gene or its cDNA involves complex isolation and purification of protein for antibody production, as well as lengthy screening procedures of an expression library.[4,5] Alternatively, availability of cDNA as a probe from heterologous systems might expedite the gene isolation process but involves elaborate, time-consuming library screening protocols. Here, we have used a strategy for fast cloning of SS cDNA of sugarcane using an anchored polymerase chain reaction (APCR) protocol. Such an approach might be applicable to future cloning of the other genes for enzymes important in sucrose metabolism.

In PCR amplification, primer extension by DNA polymerase is used to make a copy of a cDNA strand, which can then serve as a template for extension for a second primer in the opposite orientation. Multiple rounds of this process lead to exponential accumulation of the sequence of interest.[6] The result of the PCR is a DNA fragment of defined length with known oligonucleotide sequences incorporated into its ends. PCR has been used extensively for amplifying target sequences,[7] for directing mutagenesis by inserting nucleotide changes in primers,[8,9] by overlap extension,[10,11] or by gene SOEing[12] and for quantifying gene expression.[13,14] PCR has also been used to amplify and clone genomic[15] and full-length cDNAs.[16–22] cDNAs with sequences outside the priming sites were obtained by adding a universal priming site by APCR.[23]

In this study, we report the cloning of SS cDNA of sugarcane by PCR, based on published sequence information.[24,25] We first identified the conserved regions in SS sequence (of maize and wheat) and prepared oligonucleotide primers (R1 and R2) complementary to sense and antisense strands. Amplification with these two primers would replicate only internal sequences of SS. Hence, we have used these primers, one at a time, in an APCR protocol to extend amplification to the ends in two separate reactions. We then excised the overlapping regions of the two (5′ and 3′) amplification products and religated the non-overlapping DNA sequences to yield a full-length cDNA. We sequenced the resulting construct and compared it with published SS sequences from heterologous systems to confirm that the cDNA is a complementary copy of the SS gene.

MATERIALS AND METHODS

Oligonucleotide Primers

Two of the SS published sequences in maize[24] and wheat[25] were aligned from the 5′ to 3′ ends of cDNA. Two sequences with more than 95% homology between maize and wheat were selected to synthesize oligonucleotide primers specific for the SS gene. The first se-

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quence of 16 bp (R1) toward the 5′ end of cDNA was 916 bp upstream, while the second sequence of 18 bp (R2) was 245 bp upstream from the polyadenylation signal of maize Sh gene (Table 1). Complementary deoxynucleotide sequences were synthesized as primers with an Applied Biosystems Model 380B DNA synthesizer (University of Hawaii Biotechnology Center). Primers were detritylated by treating with 90% acetic acid followed by repeated co-evaporation with alcohol. The hydrolyzed trityl oligonucleotides were then desalted by passing through Sep-Pak chromatography columns.

RNA Isolation and cDNA Synthesis

Total RNA[26] and poly(A)$^+$ RNA (Message affinity paper, Amersham) were prepared from sugarcane cell cultures of *Saccharum* spp. hybrid cv H50-7209. Poly(A)$^+$ RNA (5 μg) was primed with oligo(dT) and R2 primers, in two separate reactions, and reverse-transcribed using Molony murine leukemia virus (Mo-MLV) reverse transcriptase according to the manufacturer's instructions (Stratagene). The second-strand cDNA was synthesized from oligo(dT)-primed cDNA using the RNase H method.[27] Both single- and double-stranded cDNAs were extracted twice with phenol and chloroform. Unincorporated nucleotides were removed by passing the cDNA twice through a CL4B Sepharose column and repeated precipitations with ethanol.

Homopolymeric Tailing and Amplification of 5′ (SSp1) and 3′ (SSp2) cDNAs

An oligo(dC) tail was added to the 3′ ends of single-stranded cDNAs using terminal deoxynucleotidyl transferase in 50-μl reaction volume.[28] Addition of the (dC) tail was monitored by incorporating ^{32}P-labeled dCTP and counting the TCA precipitable counts. Optimal incubation period and concentration of cDNA were determined by conducting a time course and concentration experiment. A 30-min incubation period with 500 ng of cDNA was selected as an optimum and was used in subsequent experiments. Terminal transferase enzyme was heat-inactivated and the reaction mix was used directly in PCR experiments. The R2 and oligo(dG) primers were used for amplification of 5′-related sequences yielding a product labeled as SSp1. The R1 and oligo(dT) primers were used to amplify 3′ end sequences to produce SSp2. PCR reactions were carried out in 100-μl volumes with 2.5 mM of each dNTP and 100 pmoles of respective primers using an Amplitaq PCR kit (Perkin-Elmer Cetus). The PCR reaction mix was exposed to ultraviolet (UV) light (300 nm) for 1 min to eliminate contaminants before adding DNA and *Taq* polymerase. The negative controls, such as DNA without primers and PCR reaction without target DNA, were used with each experiment to identify possible contaminants. Twenty cycles of PCR were run in a Perkin-Elmer Cetus model 480 thermocycler with the following conditions for each cycle: denaturation (94°C) for 1 min, annealing (50°C) for 2 min, and extension (72°C) for 3 min. The SSp1 and SSp2 cDNAs amplified with only 10 cycles of PCR were cloned into a plasmid vector PCR 1000 according to the manufacturer's instructions (In Vitrogen). Double-stranded DNAs of SSp1 and SSp2 were sequenced using M13 reverse and forward primers by the dideoxy chain-termination method.[29]

Restriction Mapping and Construction of Recombinant cDNA

Amplified products were purified with phenol:chloroform extraction and ethanol precipitation. PCR-amplified cDNAs were restriction mapped by single and double digestions with six different restriction enzymes, *Bgl*II, *Hin*fI, *Kpn*I, *Nar*I, and *Nco*I (BRL), to locate overlapping restriction sites. Both SSp1 and SSp2 DNAs were digested with *Nar*I restriction enzyme and separated on agarose gel. The DNAs of 1.7 kb (SSp1) and 650 bp (SSp2) were purified from the agarose. A hybrid molecule, representing a complete SS cDNA of sugarcane, was obtained by ligating the SSp1 and SSp2 purified fragments with T4 DNA ligase according to the manufacturer's instructions (Stratagene). For sequencing, the cDNA was subcloned into a pGEM4Z (Promega) expression vector by blunt-end ligation.[30] Double-stranded cDNAs from three independent clones of each SSp1 and SSp2 were sequenced by the dideoxy chain-termination method using M13 forward and reverse primers.[29]

Southern Blot

High-molecular-weight DNA, prepared from the young unexpanded leaves of var. Badila, was digested with *Bam*HI, *Eco*RI, and *Hin*dIII restriction enzymes.[28] DNA was then electrophoresed in a 0.8% agarose gel and transferred onto Duralose membrane (Stratagene) and was hybridized with PCR-reconstructed, random-primed SS cDNA probe.[30] Hybridized blots were washed at high stringent conditions, twice with 2.5× SSC, 0.1% SDS at 65°C, and twice with 0.1× SSC, 0.1% SDS at 65°C for 15 min each.

RESULTS

The experimental method used to isolate SS cDNA of sugarcane is outlined in Figure 1. To design the primers for amplifying the core region of SS cDNA, we first identified the regions of high amino acid sequence conservation among the previously sequenced SS genes.[24,25] We then synthesized oligonucleotide primers of 16 (R1) and 18 (R2) bases that were complementary to the conserved sequences (Table 1). *Bam*HI linkers were added to the 5′ end of primers to facilitate cloning of amplified cDNA.

Amplification of Core Sequences

Poly(A)$^+$ RNA of cell cultures was used

TABLE 1 Sh-5′ and Sh-3′ Indicate the Primers Complementary to 5′ and 3′ Ends of SS cDNA, Respectively

	PCR primer sequences
Sh-5′: R1	5′-GCGCGTCTCGACCGCG-3′ (746)
BSh-5′:	5′-CTGGATCCGCGCGTCTCGACCGCG-3′
Sh-3′: R2	5′-CGGTACTCTAGGGCGTAG-3′ (145)
BSh-3′:	5′-CTGGATCCCGGTACTCTAGGGCGTAG-3′

The prefix B at the left indicates the primers with *Bam*HI linkers. Underlined bases denote the *Bam*HI sequences added to the 5′ end. In the text Sh-5′ was referred to as R1, and Sh-3′ was referred to as R2.

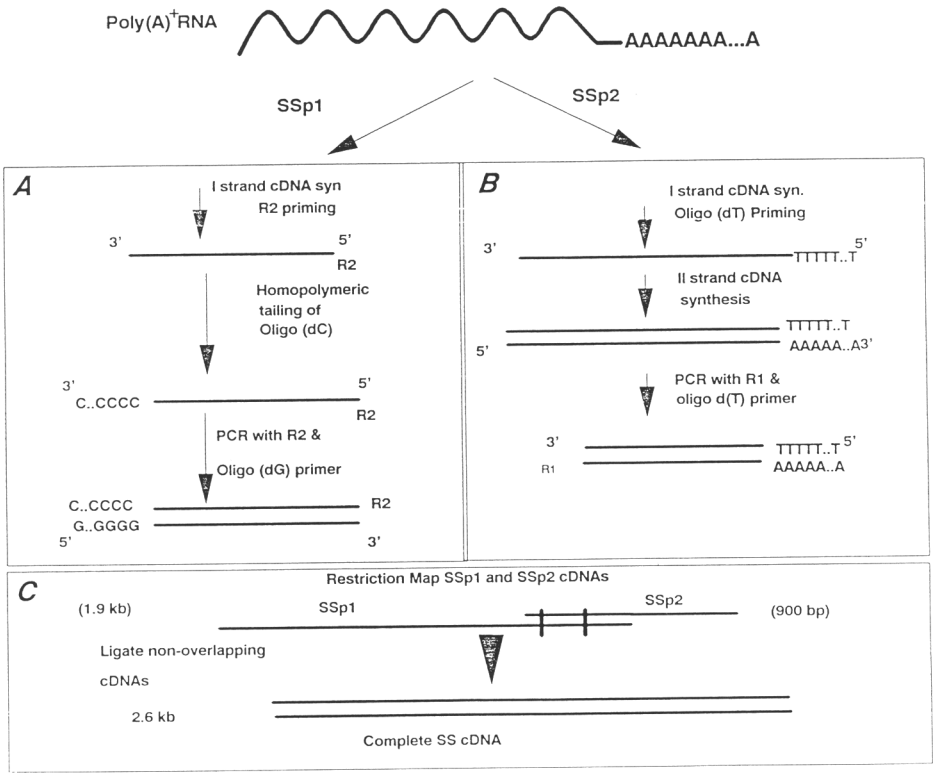

FIGURE 1 Schematic representation of the three-part strategy for SS cDNA amplification. (*A*) Steps involved in the amplification of 5′ end sequences (SSp1) of SS cDNA using R2 and oligo(dG) primers. (*B*) Amplification of 3′ end sequences (SSp2) using R1 and oligo(dT) primers. (*C*) Restriction mapping of SSp1 and SSp2 DNAs and construction of complete SS cDNA by ligating non-overlapping fragments.

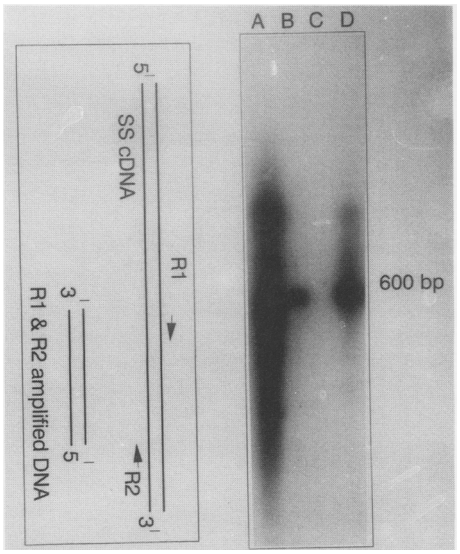

FIGURE 2 An autorad showing the results of amplification of SS internal sequences. A 600-bp region of SS using R1 and R2 primers. (Lane *A*) Sugarcane genomic DNA; (lane *B*) cDNA from cell cultures of sugarcane; (lane *C*) a negative control without sugarcane cDNA; (lane *D*) maize control with the same primers. Number on the right represents size of amplified products and on the left the schematic representation of amplified region.

to synthesize target cDNA for PCR amplification because the SS transcript pool was more abundant in cell cultures than in other tissues (data not shown). The R1 and R2 oligonucleotide primers spanned 600 bp in wheat and maize and amplified cDNA of same size in sugarcane (Fig. 2). The purified 600-bp cDNA was subcloned into *Bam*HI sites of the pGEM4Z vector and was sequenced, using either SP6 and T7 primers or R1 and R2 primers, and was compared with maize Sh1 sequences. The amplified DNA shared more than 93% sequence homology with that of maize (Fig. 3), indicating that the primers indeed amplified SS cDNA.

Amplification of 5′ (SSp1) End Sequences

To amplify the 5′ end of SS cDNA we first synthesized target cDNA from R2 primed cell culture mRNA and added oligo(dC) at its 3′ end using terminal deoxynucleotidyl transferase. A 1.9-kb DNA fragment (SSp2) was obtained when homopolymeric tailed cDNA was amplified with equimolar concentrations of specific R2 and nonspecific oligo(dG) primers (Fig. 4A). Purification and further amplification of the products increased the specificity of the band and resulted in a discrete band of amplified DNA (Fig. 4A).

Amplification of 3′ (SSp2) End Sequences

The 3′ sequence of the SS cDNA was amplified by using equimolar amounts of R1 and oligo(dT) primers. After 20 cycles of amplification, we observed a faint band of 800-bp size within a continuous smear. To minimize misincorporation of nucleotide bases, the PCR products after 10 cycles were used to clone into the PCR-1000 plasmid vector. For proper visualization of specific amplified products, DNA of 800 bp was gel-purified and used for reamplification. A single band of 800 bp was observed in the PCR amplification of size-selected DNA fragments (Fig. 4B).

Restriction Mapping of 5′ (SSp1) and 3′ (SSp2) Amplified SS cDNAs

The SSp1 and SSp2 restriction maps (Fig. 5) indicate the presence of common and unique restriction sites in their PCR-amplified cDNAs. Restriction sites *Hin*fI, *Nco*I, and *Nar*I were present in both clones. *Kpn*I restriction site was unique to SSp1 cDNA and a *Bgl*II was identified only in SSp2 cDNA. The *Nar*I common restriction site was located on overlapping sequences of SSp1 and SSp2. SSp1 cDNA was cut into ~150- and 650-bp cDNA fragments, whereas SSp2 DNA was divided into ~1650 and 250 cDNA segments when it was digested with *Nar*I restriction endonuclease. The 650- and 1700-bp cDNAs were purified and ligated to each other using T4 DNA ligase (Fig. 6) to synthesize a complete cDNA of sucrose synthase.

The amplified and ligated SS cDNA was sequenced using the pGEM4Z vector-specific primers. The sequence included 18 bp of poly(T:A) at the 3′ end and 16 bp of poly(G:C) at the 5′ end. The remaining sequence was conserved (~87%) when compared with earlier published maize Sh1 sequences.

```
   GCG CGT CTC GAC CGC GTG AAG AAT ATG ACT GGG CCG GTC   39
 1 Ala Pro Leu Asp Arg Val Lys Asn Met Ttr Gly Pro Val

   GAG ATT TCC GGC AAG AAG GCG CGC CTA AGA GAG CTG GCG   78
14 Glu Ile Ser Gly Lys Lys Ala Arg Leu Arg Glu Leu Ala

   AAT CCC GTG ATC GTC GCC GGC GAC CAT GGC AAG GAG TCC  117
27 Asn Pro Val Ile Val Ala Gly Asp His Gly Lys Glu Ser

   AAA GAC AGG GAT GAG GCC GAG GAG CAG GGC GGA TTT AAG  156
40 Lys Asp Arg Asp Glu Ala Glu Glu Gln Gly Gly Phe Lys

   AAG ATG TAT AGC CTC ATT GAC GAC TAC AAG TTC AAG GGG  195
53 Lys Met Tyr Ser Leu Ile Asp Asp Tyr Lys Phe Lys Gly

   CAT ATC CGC TTG ATC TCG GCG CAG ATG AAC CGC GTC CGC  234
66 His Ile Arg Leu Ile Ser Ala Gln Met Asn Arg Val Arg

   AAT GGG GAG CTG TAT CAG TAC ATT TGC GAT ACT AAG GGG  273
79 Asn Gly Glu Leu Tyr Gln Tyr Ile Cys Asp Thr Lys Gly

   GCA TTC GTA CAG CCG GCG TAC GAA GCT TTT CGG CTT GAT  312
92 Ala Phe Val Gln Pro Ala Tyr Glu Ala Phe Arg Leu Asp

   TGC GAT CGA GTC CAC GAA GTG CGG TCT GCA AAG GAT CGC  351
105 Cys Asp Arg Val His Glu Val Arg Ser Ala Lys Asp Arg

   GAC CTG CCA TGG CGC CCA TGT GAG ATC ATC GCT GAT GGG  390
118 Asp Leu Pro Trp Arg Pro Cys Glu Ile Ile Ala Asp Gly

   GTG TCT GGC CTG CAC ATT GAC CCG TAT CAT AGC GAT AAA  429
131 Val Ser Gly Leu His Ile Asp Pro Tyr His Ser Asp Lys

   GAC GCC GAT ATC CTG GTC AAC TTT TTT GAT AAA TGC AAT  468
144 Asp Ala Asp Ile Leu Val Asn Phe Phe Asp Lys Cys Asn

   GCA GAT CCT AGC TAC TGG GAC GAG ATC TCG CAA GGT GGC  507
157 Ala Asp Pro Ser Tyr Trp Asp Glu Ile Ser Gln Gly Gly

   CAG AGA ATT TAT GAG AAA TAC ACC TGG AAG CTC TAT TCA  546
170 Gln Arg Ile Tyr Glu Lys Tyr Thr Trp Lys Leu Tyr Ser

   GAA AGG CTG ATG ACA CTT ACC GGC GCA TAC GGG TTT TGG  585
183 Glu Arg Leu Met Thr Leu Thr Gly Ala Tyr Gly Phe Trp

   AAT TAC GTG AGT AAA CTG GAG AGG GGG GAT ACC CGT TAC  624
196 Asn Tyr Val Ser Lys Leu Glu Arg Gly Asp Thr Arg Tyr

   ATC GAT ATG TTC TAC GCC CTT GAG TAC CCG 654
209 Ile Asp Met Phe Tyr Ala Leu Glu Tyr Pro
```

FIGURE 3 Nucleotide sequence of sucrose synthase gene, amplified with R1 and R2 primers, and its deduced amino acid sequence. Nucleotides are numbered on the right; amino acids are numbered on the left. Overlapping *Nar*I restriction site in the center and primer sequences are underlined. The above sequence begins at base 1028 of reconstructed cDNA.

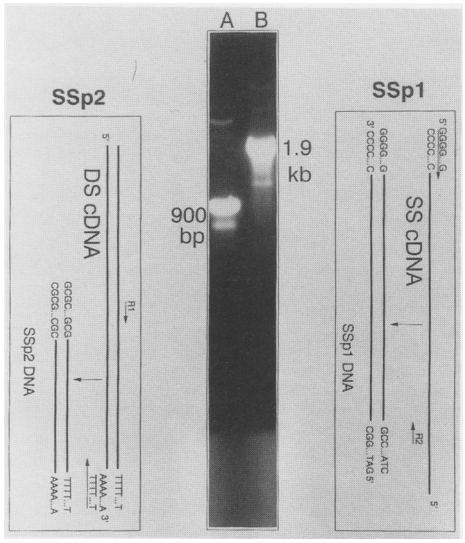

FIGURE 4 Agarose gels stained with ethidium bromide showing the results of amplification of SS 5′ and SS 3′ end sequences. A 1.9-kb SSp1 cDNA amplified with oligo(dG)$_{20}$ and R2 primers (*A*), and SSp2 cDNA (900 bp) amplified with R1 and oligo(dT) primers (*B*). Also shown are diagrammatic representations of amplified regions from an SS cDNA.

A Southern blot of sugarcane genomic DNA hybridized with our PCR-reconstructed probe showed single *Bam*HI and *Hin*dIII fragments of 10.8 kb, whereas a strong 3.6-kb band and a less intense band of 3.8-kb restriction fragments were observed in *Eco*RI digests (Fig. 7).

DISCUSSION

Lack of an antibody to the protein and lack of a complementary cDNA as probe to the gene are often barriers to cloning of genes of choice. At other times either the antibody or the cDNA probe from a heterologous source may not cross-react in a particular system because of limited homology between the heterologous genes. For example, the antibodies for SPS in spinach did not cross-react with the SPS antigen of maize, sugar beet, or soybean.[31] Sequence comparisons for particular genes in different systems identified short consensus regions, referred to as sequence tagged sites (STS). The STS are mostly unique to the given gene and can be used to isolate the desired gene sequence from any species that contains it.[32] STS for SS were identified in internal sequences of maize[24] and wheat[25] cDNAs. The present strategy takes advantage of those conserved nucleotides or STS identified from the sequence data of SS cDNAs of maize and wheat.

Given the choice of our R1 and R2 priming sites, it was not possible to amplify full-length SS cDNA in a single step. However, we added nonspecific oligo(dC) priming sites at the 5′ end and used universal oligo(dT) primer at the 3′ end to amplify the regions outside the specific internal priming sites. Separate amplifications resulted in two DNA segments, one containing the 3′ end (SSp2)

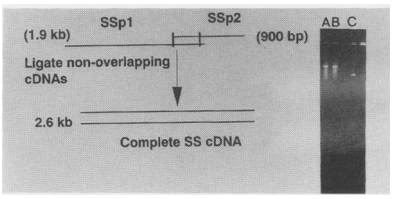

FIGURE 6 Ethidium bromide-stained agarose gel showing a 2.6-kb reconstructed SS cDNA after eliminating overlapping segments (lane *C*) and after ligating with pGEM4Z plasmid (lanes *A* and *B*).

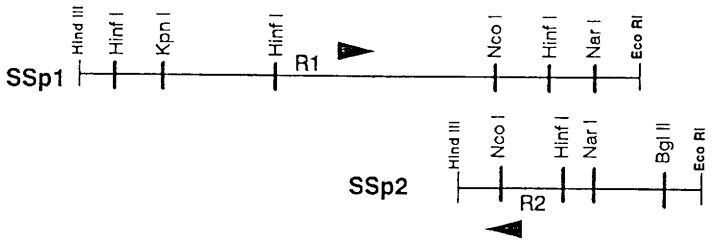

FIGURE 5 Restriction map of SSp1 and SSp2 containing regions of PCR-amplified SS cDNA to identify overlapping restriction sites.

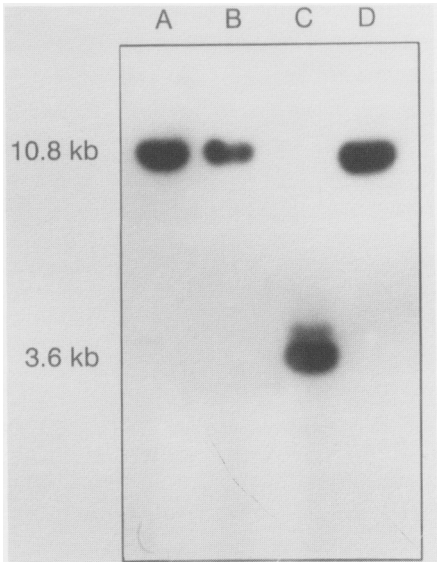

FIGURE 7 Genomic Southern blot of sugarcane hybridized with PCR-reconstructed cDNA as probe. Each lane contained 5 μg of DNA isolated from young unexpanded leaves of var. Badila (*Saccharum officinarum*) and digested with *Bam*HI (lane *A*), *Hin*dIII (lane *B*), *Eco*RI (lane *C*), and maize kernel DNA (lane *D*) digested with *Bam*HI.

and the other consisting of 5′ end (SSp1) of SS. Similar techniques of APCR were employed to amplify small sequences of ~800 bp of the tropomyosin gene[33] and to construct a mouse oocyte cDNA library.[34] In these two cases, the primers were developed from homologous sequence information and used to amplify 5′ sequences. Our complete SS cDNA was reconstructed by identifying and removing overlapping restriction sites and ligating non-overlapping DNA fragments. A similar technique of gene construction referred to as overlap extension or gene SOEing was used to introduce base changes in internal sequences of DNA.[11,12] In the above experiments, two DNA segments with little common sequence were annealed and *Taq* polymerase was used to extend into the non-overlapping regions.

Taq polymerase does not have proofreading ability and accumulates 0.3–0.8% base changes in 20–30 cycles of PCR.[35] Our experiment involved the cloning of amplified PCR products within 10 cycles to reduce the percentage of error in base incorporation. Three independent clones of SSp1 and SSp2 were sequenced to detect percentage of misincorporation of base pairs in amplified cDNA. Sequences of all three clones were 100% homologous, ruling out the possibility of wrong base pair incorporation. PCR amplification is also known to become less efficient with an increase in product size.[36] Our PCR reconstruction avoids direct amplification of the entire 2.8-kb SS transcript by separately amplifying the 5′ and 3′ ends of the gene. However, we cannot completely rule out the possibility of minor base changes by our protocol.

Separate amplifications of 3′ and 5′ sequences might also create a problem of constructing a recombinant molecule between two different genes. The presence of 100% homology in overlapping regions of SSp1 and SSp2 indicates that the amplified regions are the products of the same gene, and reconstruction of DNA from such amplification should not have generated a hybrid DNA molecule.

Although the protocol described here is faster and more efficient than traditional library construction and screening, we recognize that PCR may generate false constructs. This problem can rise in a variety of ways and potentially can cause several problems. Amplification of false positives could arise from: (1) introduction of an exogenous DNA source, (2) contamination of sample RNA with its own or external DNA source, (3) introduction of base changes in the primers synthesized from heterologous sequence information, or (4) amplification of unrelated DNAs due to homology of the primers to those unrelated target sequences. We have attempted to avoid the introduction of an exogenous DNA by exposing the reaction mixture to UV light to destroy foreign DNA before adding our selected DNA and *Taq* polymerase. It has been reported that UV irradiation of PCR mixtures before adding target DNA can eliminate unwanted amplifiable DNA contaminants.[37] Care was exerted to avoid contamination of false positives in sample preparation and PCR amplification as recommended.[38] Contamination of sample DNA in RNA preparation would amplify a different size DNA fragment because of the presence of an intronic sequence between R1 and R2 primers. We did not observe a different size fragment in any of our PCR amplifications.

We tried to minimize the incorporation of base changes in the amplified and reconstructed SS cDNA of sugarcane in two ways: amplifying the SS cDNA in two separate steps (SSp1 and SSp2) and by eliminating R1 and R2 priming sites while reconstructing target DNA. R1 and R2 primers were synthesized based on maize sequences, which may be different from sugarcane sequences. Finally, presence of a single band in different reactions and their sequence homology with published SS gene sequences[24,25] rules out the possibility of the amplification of unrelated sequences.

The results demonstrate the utility of PCR for rapid cloning of relatively large cDNAs which are previously characterized in heterologous systems.

ACKNOWLEDGMENTS

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Sequence data is recorded as EMBL/GenBank Z11532.

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