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cDNA-SCoT: A novel rapid method for analysis of gene differential expression in sugarcane and other plants

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Abstract

Gene expression analysis is extremely important in the field of agriculture. The correct analysis could provide theoretical and scientific references for wide-range genetic improvements in plants to increase crop production quality and quantity. To correctly analyze gene expressions, a high-performing, effective, and inexpensive gene expression method is needed. Throughout all previous relevant researches, many methods were created for the detection and analysis of differentially-expressed genes in plants. Although these methods are technologically advanced, each marker system has its own disadvantages when used for gene expression analysis. All these methods have been created in various laboratories for different application purposes. In addition, each method is highly influential to different crop species, technical expertise, available equipments, available research funding, etc. which are not always available. So, these methods are unsuitable for a pure gene expression analysis. To simplify the gene expression analysis without the outside influences of crop species differentiation, different technical skills and equipments, and limited funding, we developed a new method for gene differential expression in plants based on the start codon targeted polymorphism (SCoT) DNA Marker technique, called cDNA-SCoT. The cDNA-SCoT is advantageous compared to all other existing methods because it is relatively more efficient, faster, cheaper, simpler to operate, and the results can be easily reproduced. This method also does not require expensive machineries or complicated chemical mixtures to operate. Consequently, this newly created method was successfully tested on sugarcane genes through this study. We predict that, due to its beneficial qualities in differential gene analysis in comparison to other methods, the cDNA-SCoT would play a more important role in studying differentially expressed genes, finding new genes, investigating the molecular mechanism of resistance, and more.

Keywords: cDNA-SCoT, differential gene expression, molecular marker, gene.
Abbreviations: cDNA-SRAP_sequence-related cDNA-amplified polymorphism; cDNA-SCoT_cDNA-amplified start codon targeted; cDNA-AFLP_cDNA-amplified fragment length polymorphism; DDT_DL-dithiothreitol; TDFs_transcript-derived fragments.

Introduction

Stepping along with the improving developments in the field of molecular biology, many new promising alternative marker techniques have emerged. Currently, many approaches have been developed for the analysis of differential gene expression at the mRNA level in various plants or animals cells. Some of the most notable methods used to detect differentially-expressed genes in plants include mRNA differential display (DD), Representational difference analysis (RDA), suppression subtractive hybridization (SSH), serial analysis of gene expression (SAGE), cDNA-AFLP, cDNA-SRAP, cDNA microarray, etc. (Velculescu et al., 1995; Bowler, 2004; Blackshaw et al., 2007; Hillmann et al., 2007; Huang et al., 2007a, b; Liang et al., 2007; Nettuwakul et al., 2007; Polesani et al., 2008; Wee et al., 2008; Hillmann et al., 2009; Zamharir et al., 2011; Xu et al., 2011). However, regardless of the abundant quantity of available methods, there exist both advantageous and disadvantageous traits for each system. For example, differential display (DD) is a method with simple operations but the results show poor consistency, in which the coding region is also difficult to amplify (Ledakis et al., 1998). The cDNA-AFLP method produces more consistent results compared to that of DD, but it is considerably more complicated to operate. In order to perform this method, several steps are involved, which include reverse transcription, digestion, ligation, and amplification. Preparation of initial conditions is extremely difficult to sort out. In addition to the complex operations, the cDNA-SRAP method has much more problems, including primers overflow (more than 1000 primer combinations), time consuming procedures, and low results repeatability. Despite their flaws, the above techniques, along with some other more notable methods mentioned in the beginning are widely applied to different fields in different laboratories. They can be adapted and used for different crop species to develop genome mapping, gene tagging, genomic

and cDNA fingerprinting (Li and Quiros, 2001; Collard and Mackill, 2009).

The relatively new SCoT method is a PCR marker technique based on the translation of start codon which is simple and reliable. By applying the SCoT method, the PCR amplification using the designated single primer can generate dominant polymorphic markers with a predisposition towards the candidate functional gene region. Therefore, the SCoT method can be widely applied to various study fields in different laboratories (Xiong et al., 2009). After numerous applicable researches from different labs, the SCoT polymorphism marker technique has been successfully applied in rice (Collard and Mackill, 2009), peanut (Xiong et al., 2010), longan (Chen et al., 2010), citrus (Han et al., 2011), *Vitis vinifera* (Zhang et al., 2011), tree peony (Hou et al., 2011), persimmon (Deng et al., 2012), etc.

One of the main purposes of all the above mentioned methods is to search for the differentially expression in plant and to provide valuable information to determine the gene functions. However, although technologically advanced, each marker system has certain shortcomings when it comes to differential gene analysis, such as producing too much differential bands, high expenses, high false positive rate, low sensitivity, poor repeatability, technical difficulties, etc., depending on the selected method.

Also, since these methods were created for various application purposes other than gene expression analysis from multiple backgrounds, the methods are highly susceptible to varying crop species, technical expertise, available equipments, and available research funds. Therefore, in order to create a gene expression analysis technique that avoids all the flaws in the existing methods, the cDNA-SCoT was recently developed in our lab. This novel method is highly efficient, rapid, economical, simple to operate, and produces reproducible results, which suits our research purposes perfectly.

Results and discussion

Principles of the method

Primer Designs

The SCoT technique is based on the single primer amplified region principle since it uses a single primer as both the forward and reverse primer, similar to that of the RAPD or ISSR technique. However, PCR amplification using SCoT primer targets the gene regions surrounding the ATG initiation codon on both DNA strands (Collard and Mackill, 2009). The cDNA-SCoT primers in this study were designed based on Collard and Mackill's design (2009), in which the specific nucleotides in the primer sequence were fixed as follows: the ATG codon fixed at positions +1, +2, and +3, 'G' fixed at position +4, 'C' fixed at position +5, and 'A', 'C', and 'C' fixed at positions +7, +8, and +9, respectively. Most primers differed from each other by at least one nucleotide with an emphasis on variations at the 3' end, which is critical for primer construction. Therefore, we designed 10 more single primers based on that primer construction principle (Table 1).

PCR conditions

In our study, the conditions of PCR, based on Collard and Mackill's principle of the SCoT marker (2009), suggest that the total RNA was isolated from young stem in sugarcane and reversed to cDNA. Then the first-strand cDNA, second-strand cDNA, purified product of the first-strand cDNA, and purified product of the second-strand cDNA were all used as templates for the amplification process after the optimization of the amplification system, which has been successfully applied in sugarcane for the first time through this study. The research data results show that identification of differential gene expression by cDNA-SCoT is reasonable. In sum, the cDNA-SCoT method is determined to be a highly efficient method with data reproducibility that is both time-saving and economical. Thus, the cDNA-SCoT technique is complementary to the conventional cDNA-AFLP method and cDNA-SRAP method.

Different expression patterns of cDNA-SCoT

After the RNAs were extracted from young stems of sugarcane, single-stranded and double-stranded cDNAs were synthesized for amplification. The results suggest that the purified product of the second strand cDNA was the best amplification template. At the same time, after optimizing the PCR system, the data specificity was tested by amplifying different selected samples and the system sensitivity was examined by operating the serial dilution method. The best results were obtained by using 10 times diluent of the purified product using the second strand cDNA as template after 35 cycles of amplification. Most of the primers could produce three to fifteen bands through the amplification process ranging from 100 to 1200 bp, which could be noted by visual observation (Fig. 1). The results indicated that the cDNA-SCoT processing exhibits the following characteristics:

(1) The conservative sequences of cDNA-SCoT primers are already given from previous research results. So, the primer design is simple. More new primers can be designed based on the original primer sequence after slight modification such that the cDNA-SCoT primers can be used in different species.

(2) cDNA-SCoT is based on the single amplified primers and the primer combinations. We need only 46 single primers in this present research (Table 1), which is significantly less than those primers needed for both the cDNA-AFLP and cDNA-SRAP (each method will need over 100 primers); therefore, making cDNA-SCoT's technical system easier to establish.

(3) The cDNA-SCoT can be easily used to analyze the overall levels of the cell and tissue transcriptions at the same time, which is especially important for differential expression analysis, new genes exploration, and molecular resistance mechanism investigation since this method is time-saving and easy to operate.

(4) The cDNA-SCoT can be used to compare the differential expressions in more than two different sources of mRNA samples at same time, and it can also be used for real-time quantitative analysis on countless gene products. Thus, this method is valuable for its remarkable abilities to detect gene expression in continuity analysis.

(5) When the genome is in low abundance, the cDNA-SCoT can be used for transcript analysis in which maximal gene expression information can be collected. Therefore, this method is fast and efficient to study gene expression for large-scale gene expression researches.

Note: If the isolated labeling DNA probes are used for the hybridization test, no further purifications are necessary. An aliquot of the eluted probe can be used directly in the hybridization reaction. As an optional phenol, the chloroform extraction may be performed. However, do not extract the DNA with phenol if the DNA probe is labeled with digoxygenin because the DNA will separate into the organic phase. Also, a standard etahanol precipitation with carrier (glycogen, tRNA, or linear acrylamide) may be performed for further clean-up.

Table 1. cDNA-SCoT single primer sequence**.**

No.	Primer sequence	No.	Primer sequence
1	CAACAATGGCTACCACCA	24	CACCATGGCTACCACCAT
2	CAACAATGGCTACCACCC	25	ACCATGGCTACCACCGGG
3	CAACAATGGCTACCACCG	26	ACCATGGCTACCACCGTC
4	CAACAATGGCTACCACCT	27	ACCATGGCTACCACCGTG
5	CAACAATGGCTACCACGA	28	CCATGGCTACCACCGCCA
6	CAACAATGGCTACCACGC	29	CCATGGCTACCACCGGCC
7	CAACAATGGCTACCACGG	30	CCATGGCTACCACCGGCG
8	CAACAATGGCTACCACGT	31	CCATGGCTACCACCGCCT
9	CAACAATGGCTACCAGCA	32	CCATGGCTACCACCGCAC
10	CAACAATGGCTACCAGCC	33	CCATGGCTACCACCGCAG
11	AAGCAATGGCTACCACCA	34	ACCATGGCTACCACCGCA
12	ACGACATGGCGACCAACG	35	CATGGCTACCACCGGCCC
13	ACGACATGGCGACCATCG	36	GCAACAATGGCTACCACC
14	ACGACATGGCGACCACGC	37	CAACAATGGCTACCAGCG
15	ACGACATGGCGACCGCGA	38	AAGCAATGGCTACCACCG
16	ACCATGGCTACCACCGAC	39	ACGACATGGCGACCAGCG
17	ACCATGGCTACCACCGAG	40	ACGACATGGCGACCACGT
18	ACCATGGCTACCACCGCC	41	ACGACATGGCGACCGCGG
19	ACCATGGCTACCACCGGC	42	ACCATGGCTACCACCGAT
20	ACCATGGCTACCACCGCG	43	ACCATGGCTACCACCGGT
21	ACGACATGGCGACCCACA	44	GCAACAATGGCTACCACG
22	AACCATGGCTACCACCAC	45	CATGGCTACCACCGGCCG
23	CACCATGGCTACCACCAG	46	CCATGGCTACCACCGGCA

Notes: Primers #1-#36 were from Collard and Mackill (2009); primers #37-#46 were designed by us. The underlines of ATG codon in the primer sequence were fixed.

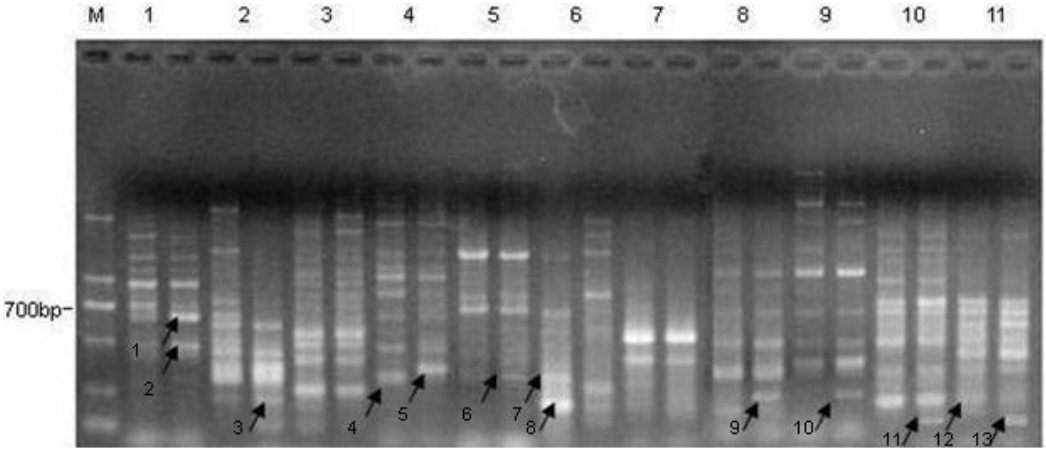

Fig 1. Amplified results of cDNA-ScoT. M: Marker (DMarker II), 1-11 show the single of 2, 5, 7, 8, 13, 15, 19, 20, 23, 28, 33 (Table 1); the template for odd was control, and the template for even was treatment by GA_3. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 represent the fragments TDFs8, TDFs11, TDFs19, TDFs13, TDFs17, TDFs2, TDFs5, TDFs7, TDFs12, TDFs4, TDFs14, TDFs6, and TDFs21, respectively.

Table 2. Consensus sequences flanking the ATG start codon.

Consensus sequence	Nucleotide position																				
	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Monocot consensus	G	C	A/C	A/G	C/A	C	A	T	G	G	C	G	A/G	C	C/G	A/G	C	C	G	-	-
Dataset I	T	A/C	A	A	C	A	A	T	G	G	C	T	N	C	C	T/A	C	N	A	C/T	A/C
Dataset II	N	N	N	G/A	A	N	A	T	G	G	N	G	N	N	G	N	N	A	N	A	N

Monocot consensus reference by Joshi et al. (1997),
Dataset I was based on highly expressed genes described in Sawant et al. (1999),
Dataset II was based on lowly expressed genes described in Sawant et al. (1999),
Table 1 Reference by Collard and Mackill. (2009).

Materials and methods

Plant materials

Cane sets of sugarcane (*Saccharum officinarum* spp. cv. ROC22.) were planted in barrels in the greenhouse at Guangxi University for 9 months. Our purpose was to elongate the sugarcane stems to improve the plant's growth and increase production quality. Although spraying sugarcane plants with gibberellic acid (GA_3) has been confirmed to promote sugarcane internode elongation, GA_3's processing procedures on sugarcane are still unclear. Therefore, differential gene expressions in sugarcane under GA treatment were investigated in order to obtain some fragments of the differentially expressed genes associated with sugarcane stem elongation to study GA_3's processing procedures.

The plants were sprayed with 200 mg of GA_3. Plants sprayed with GA_3 at early elongation stage were used as treatment, while the plants sprayed with distilled water were used as control. Young plant stalks (the part from apical meristem to internode +1) of 6 sugarcane plants were sampled at 0 h, 6 h, 12 h, 24 h, and 48 h after treatment, respectively.

Primer design

Primer design is the most important part of SCoT analysis. This primer design was referenced from the consensus sequences derived by Joshi et al. (1997) and Sawant et al. (1999). For the ATG codon (+1, +2, and +3) in the primer design, 'G' was fixed at the position +4, and 'A', 'C', and 'C' were fixed at the positions +7, +8 and +9, respectively. All the primers were 18-mer and the GC content ranged between 50% and 72% (Collard and Mackill, 2009). We designed 10 new primers (Table 1, No. 37-46) based on the principle of primers design (Table 2).

Total RNA extraction

Total RNAs were extracted from the young stalks using isothiocyanate (Wu et al., 2009). Then the RNAs were measured by determining ratios of OD260/28 and OD260/230, and agarose gel electrophoresis was used for their reliability analysis. The concentrations of RNA from different treatments were regulated to equal amounts. Lastly, equal amounts of RNA of the control and treatment were mixed individually to make two sample pools.

cDNA synthesis

First strand cDNA synthesis was carried out as follows：A 0.2 ml vial was used for the mixture then 0.05-1 μg total RNA, 1 μL cDNA synthesis (CDSⅢ) primer (10 μM), and l μL SMARTTM I A Oligonucleotide (10 μM) were added into the vial. The vial was adjusted to 5 μL with sterilized ddH_2O then gently mixed and centrifuged. The vial was incubated at 72°C for 2 min, chilled on ice, spun down, and then placed back on ice. Again, 2 μL 5×first strand cDNA, 1 μL DTT (20 M), 1 μL dNTP Mix (10 M), 1 μL Power Script Reverse Transcriptase (BD Biosciences) (200 U μL-1) were added into the vial then gently mixed and centrifuged. The mixture was incubated at 42 °C for 90 min for reaction processing. Then the reaction processing was terminated by chilling the mixture on ice.

The second strand cDNA synthesis：A 0.2 ml vial was used for the mixture, then 0.5 μL of the first strand cDNA synthesis, 20.3 μL ddH_2O, 2.5 μL buffer, 0.5 μL dNTP, 0.5 μL 3' primer, 0.5 μL 5' primer, and 0.3 μL EXTaq were added into the vial. The total volume was calculated to be 25 μL. Reaction condition processing steps include an initial denaturation at 95°C for 1 min, followed by 22 cycles of 95°C for 20 s, then at 68°C for 6 min. The cDNA purification was done using the PCR Purification Kit (Qiagen) without the taq polymerase (we used EXTaq polymerase).

Amplification system

A 0.2 ml vial was used for mixing 13.70 μL ddH_2O, 2 μL 10×PCR buffer (Mg^{2+}), 0.4 μL 10 M dNTPs, 2 μL of 10 times diluted and purified product of the second strand cDNA, 1.6 μL 10 μM single primers, and 0.3 μL 5 U μL^{-1} Taq Plus DNA polymerase.

Amplification conditions

The purified product amplification of the second strand cDNA was initiated at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 50°C for 60 s, and 72°C for 90s, lastly, it was terminated at 72°C for 7 min. A 3 μL of amplification product was taken as sample for electrophoresis on 1.2% agarose to detect the differential fragments.

Differential fragments recovery

According to the results of the amplification, two methods could be used to recover the differential fragments. The first method, electrophorosis operated on agarose gel, can recover the differential fragments if the fragments can be obviously separated by simple visual observation. The second method, electrophorosis operated on propylene acryl amine, can recover the differential fragments if the fragments cannot be separated by the first method.

DNA fragment recovery from agarose gel

(1) Remove the DNA fragments obtained from electrophorosis operated on agarose gel using a razor blade or scalpel, then transfer the DNA fragments to a 1.5 ml microcentrifuge tube.
(2) For each sample of agarose removed from the gel, measure its volume and add 3 times the amount of ADB into the sample (eg, for every 100 μl of agarose gel slice, add 300 μl of ADB).
(3) Incubate the gel slice at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved. For DNA fragments >8 kb, after the incubation step, add water to the mixture for better DNA recovery. The volume of water added should be equal to the volume of the original agarose gel slice (eg, the mixture consists of 100 μl of agarose gel slice, 300 μl of ADB, and 100 μl of water).
(4) Transfer the melted agarose solution to a Zymo-Spin™ I Column in a collection tube.
(5) Centrifuge the melted agarose solution at ≥10,000× g for 30-60 seconds. Discard the flow-through materials.
(6) Add 200 μl of wash buffer to the column and centrifuge the mixture at ≥10,000× g for 30 seconds. Discard the flow-through materials. Repeat this wash step again.
(7) Add ≥6 μl of water directly to the column matrix. Place the column into a 1.5 ml tube and centrifuge it at ≥10,000× g for 30-60 seconds to elute DNA.

Poly-Gel DNA extraction procedure

(1) Transfer the gel fragments onto a nuclease-free microscope slide. Then, using a second glass slide or a nuclease-free razor, mash and pulp and the gel together completely. Carefully transfer gel pulp to a nuclease-free microcentrifuge tube and add 250 μl of Elution Buffer. This volume is usually enough to

submerge a slice 2 mm × 10 mm × 0.8 mm slide. For a larger fragment slide, carefully adjust the volume of Elution Buffer needed until all the gel is covered. Any buffer or dH_2O can be used for this procedure; however, we recommend using the Elution Buffer to prevent DNA degradation by exogenous nucleases.

(2) Incubate the gel fragment slide in Elution Buffer for 1-4 hr at 65 ^{o}C. The elution time depends on the size of the gel fragment, DNA size, and temperature of the incubation. We find that about 70% of a 100 bp DNA fragment elutes in approximately 4 hrs at 65°C. Larger fragments will take longer to elute.

(3) Transfer both the gel and the buffer into a Green Poly-Gel filter unit mounted in a sterile 1.5 ml microcentrifuge tube using a blue pipette tip with cut-end. Centrifuge the mixture at 10,000× g ^{10} minutes at room temperature to filter the eluted DNA.

Conclusion

The cDNA-SCoT method, a new gene expression analysis method developed in our lab, compared with all other differential gene analysis methods available in the current research fields. It is relatively more efficient, rapid, inexpensive, and simple to operate. Its data results are also easily reproducible. For instance, even though the cDNA-AFLP and cDNA-SRAP methods are also reliable methods for gene expression analysis, the cDNA-SCoT method is much simpler since it needs less primer combinations to operate. Because of its positive qualities, the cDNA-SCoT method will definitely play a more important role in studying differentially expressed genes, finding new genes, and investigating molecular mechanism of resistance. So, it should be provided to future researches as an additional technique option alongside with the cDNA-AFLP, cDNA-SRAP, and other relevant techniques for multifunctional plant gene differential expression applications and investigations.

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