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Differential Expression Studies in *Hydrilla verticillata*

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INTRODUCTION

Plants exhibit three major types of photosynthesis: C_3, C_4 and CAM. C_3 plants initially fix CO_2 through the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco). However, fixation of CO_2 and O_2 by Rubisco occur at the same active site. Thus there is competition between the two substrates, and when O_2 reacts it reduces photosynthetic CO_2 fixation. C_4 plants circumvent this dilemma by using an additional C_4 acid cycle that acts as a CO_2"pump", which, in conjunction with a specialized leaf structure (Kranz anatomy), results in a high CO_2 concentration in the cells where Rubisco is located. This system enhances CO_2 fixation. Hydrilla verticillata is a submersed, freshwater angiosperm that possesses the unique ability to induce a C_4 mechanism without the specialized Kranz leaf anatomy of terrestrial C_4 plants (Bowes et al, 2002). At ambient [CO_2] Hydrilla exhibits C_3 characteristics, but as CO_2 becomes limited and temperatures rise, enzymes associated with the C_4 cycle are up-regulated. This study employs the technique of differential display to identify differentially expressed genes in C_4 Hydrilla leaves. Differential expression is a powerful technique that allows a rapid screening of RNA populations from biological systems (Liang and Pardee, 1992).

MATERIALS AND METHODS

Plant material and growth conditions

Hydrilla verticillata (L.f.) Royle plants were collected from Lake Oklawaha (Putman County, FL). Plants were washed and cut to 6 cm long sprigs and maintained in 5-L aquaria with a 25ÖC, 12-h photoperiod and a PPFD of 250 mmol m^{-2} s^{-1}. From this stock, three shoots per tube were placed in 3.5 ¥ 20-cm test tubes containing 80 mL distilled water and incubated for up to 15 d under a 30ÖC/14-h photoperiod with a PPFD of 250 mmol m^{-2} s^{-1} and a 22ÖC scotoperiod. The water was changed every other day, and the pH and dissolved inorganic carbon were allowed to fluctuate with the metabolism of the plants. Samples were collected after 15 d and frozen in liquid nitrogen.

Enzyme extraction and activity measurement

Both extraction and activity determinations followed the procedures described in Magnin et al (1997). Protein was determined following the method of Bradford (1976).

Differential display

Liquid nitrogen frozen C_3 and C_4 plant samples were ground to a fine powder, and RNA was extracted according to RNeasy Plant Kit protocol, (Qiagen, USA). A DNase treatment step was included to remove chromosomal DNA contaminants. Approximately 15 mg of total RNA from each of the C_3 and C_4 tissues were used to isolate mRNA in a total elution volume of 60 µL per sample with the help of oligotex mRNA kit (Qiagen Inc., CA, USA). First strand cDNA synthesis was performed on 10 µL each of the above mRNA samples with 2.5 µM oligo dT_{12} VA in a 20 µL reaction volume containing 40 units RNAse inhibitor and 200 units of Superscript II Reverse Transcriptase (Invitrogen Corporation, CA, USA). The reverse transcription reaction was followed according to Invitrogen's protocol. Differential display PCR was performed in a 20 µL total volume per primer pair and 1 µL of the above cDNA, employing the Advantage cDNA PCR kit (Clontech, CA, USA). The PCR reaction conditions were 94ÖC-5 min, 40ÖC-5 min, 68ÖC-5 min for one cycle; 94ÖC-30 sec, 40ÖC-30 sec, 68ÖC-5 min for two cycles; 94ÖC-20 sec, 60ÖC-30 sec, 68ÖC-2 min for 23 cycles. The PCR reactions were performed in duplicate and the results were run on 1.6% (w/v) agarose gel (12 cm W ¥ 0.5 cm H ¥ 14 cm L) at 24 V for 16 h. Following this procedure, the gels were stained in ethidium bromide at 0.1 µg mL^{-1} (w/v), and the results were documented with Polaroid film. The desired fragments were excised from the gel (see below).

Design and choice of primers for differential display

Adapting the results of Consalez et al (1999), four arbitrary primers were chosen for their high efficiency in yielding large numbers of PCR products and for their selectivity for coding regions versus untranslated regions of transcripts. They are ID # 51 (TGCCGACTCTGC/G), ID # 163 (ACGTGCCCAGCA/T), ID #688 (AAGCTGCTCGCG/C), and ID # 942 (ACGCCATCGACC/G).

DNA extraction, cloning, and sequencing

Excised fragments were extracted according to QIAEX II protocol (Qiagen USA) and eluted in 8 µL of 10 mM Tris-HCl (pH 8.5). Cloning of the above fragments was performed with a TOPO-TA Cloning Kit (Invitogen, Carlsbad, CA). At least five colonies were selected and cultured overnight in LB liquid medium containing 50 µg mL^{-1} kanamycin. Plasmid isolation was performed using a SpinPrep^{tm} Plasmid Kit (Novagen, US). Plasmids were analyzed for inserts by restriction digestion followed by agarose gel electrophoresis. Sequencing of desired clones was performed by the Sequencing Core facility at the University of Florida using a Perkin Elmer/Applied Biosystems Model 373A and 377 sequencer.

Sequencing analysis

The nucleotide sequence data obtained was edited, so that only the unambiguous sequence information was included as query in the BLAST (Basic Local Alignment Search Tool) program available online at the NCBI (National Center for Biotechnology Information) web site (www.ncbi.nlm.nih.gov). The nucleotide sequences of each clone were queried against the protein database using blastx option in which query is converted into protein sequences in all six reading frames.

RESULTS AND DISCUSSION

Verification of C_3 and C_4 Plant Material

To establish the C_3 or C_4 status of the plant material, analyses of phosphoenolpyruvate carboxylase (PEPC) activity, the initial enzyme in the C_4 cycle, were performed on the Hydrilla leaves. The specific activities were 22 and 25 µmol mg-1 protein for C_3 light- and dark-harvested plant material, respectively. In contrast, C_4 plant material showed over 20-fold greater specific activities for light- and dark-harvested material of 477 and 655 µmol mg^{-1} protein, respectively.

Differential Display

A differential display technique was used to identify up-regulated or unique genes in the C_4-induced plant tissue relative to C_3 tissue. Figure 1 shows C_3 and C_4 cDNA fragments resolved on a 1.6 % agarose gel after PCR with oligo dT12 VA and the four random primers listed above. One of the primers failed to amplify any products (Lanes 4 and 5) while primer #944 shown in Lanes 2 and 3 amplified many differentially expressed fragments. The rest of the primers produced very similar patterns of fragments.

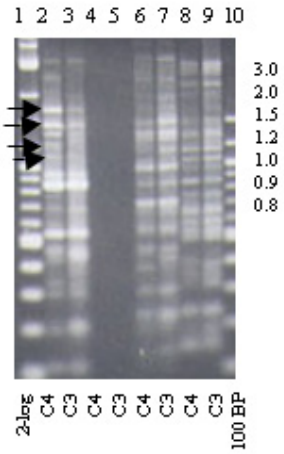

Figure 1. Differential display pattern of C_3 and C_4 cDNA with four different arbitrary primers and a single oligo-dT anchor primer in a 1.6% agarose gel electrophoresed for 16 h. Lanes 2 and 3 show fragments produced from PCR with primer #944. Lanes 4 and 5 represent primer # 688 and did not produce any fragments. Lane 6 and 7 show fragments produced with primer #163 and Lanes 8 and 9 have fragments produced from primer #51. Lane 2 shows multiple genes that are up-regulated or unique to the C_4 system and are represented with arrows. These bands were excised and subjected to PCR but failed to produce results. The fragments ranged in size from 1.0-2.0 Kb. 2-log and 100 bp molecular markers are shown on left and right side, respectively.

Figure 2 shows the reproducible results of a repeat PCR employing oligo dT_{12} VA with primer 944. The products were subjected to electrophoresis in duplicate lanes on 1.6% agarose gel under the conditions mentioned earlier. At least four unique or up-regulated bands were clearly visible in the lane where C_4 cDNA was used as template. Their sizes ranged from 0.9 to 1.75 Kb. Multiple bands were excised from positions shown on the Figure.

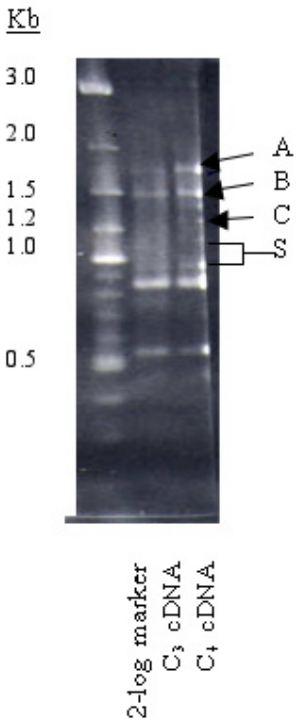

Figure 2. Repeat differential display for reproducibility. PCR product with primer #944 and anchored oligo-dT were separated on 1.6% agarose gel. Lanes with marker, C_3 and C_4 cDNA products are indicated. Arrows shown on the gel: fragments A (1.75Kb); B (1.5); C (1.3Kb); and S (0.9-1.1Kb). They were chosen as unique or up-regulated genes in the C_4 system.

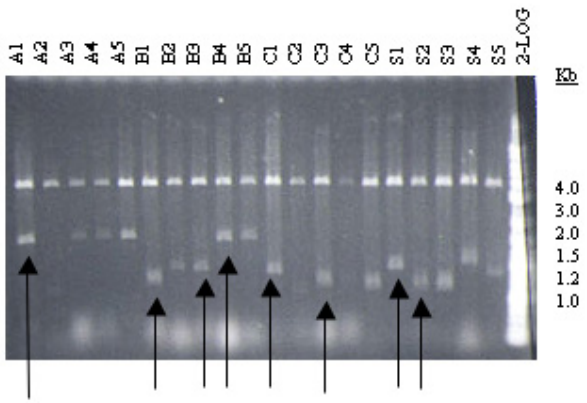

Figure 2. Repeat differential display for reproducibility.E. coli was transformed with 2 µL ligation mix (gel extracted cDNA with pCR4-TOPO vector) and spread on four LB-agarose plates with kanamycin (marked A, B, C and S), and incubated overnight at 37ÖC. Lanes A1-A5, B1-B5, C1-C5 and S1-S5 represent five independent clones selected from the corresponding plates above and grown overnight in 5 mL of LB-liquid medium with kanamycin. Plasmid DNA was isolated from each of these clones, digested with 1 unit of restriction enzyme (Eco RI) overnight at 37ÖC. Arrows indicate the clones selected for sequencing.

Sequencing

Blast searches of nucleotide sequences from C_4 fragments of unique or up- regulated genes

showed homology with entries from the Arabidopsis thaliana genome (Table 1). Three showed similarity to a putative arginine methyltransferase. The methylation of arginine residues has been implicated in the regulation of signal transduction, transcription, RNA transport, and possibly splicing. Another nucleotide sequence showed approximately 60% homology with an early dehydration response protein (ERD4) from Arabidopsis thaliana. A further sequence showed homology with a hypothetical protein that is similar to a retro element from rice (Oryza sativa). Lastly, one matched a Ser/Thr kinase protein, which has interest to us as a regulatory protein for the PEPC enzyme.

Table 1

Results of BLAST Query

Each nucleotide sequence was tranlated into protein sequences in all six reading frames. The tranlated protein products were then compared against the NCBI protein databases

Clone	Fragment length* (Kb)	Forward sequence[1] (bp)	Reverse sequence[2] (bp)	Most related sequence	E value[3]
A1	~2.0	663	705	gi|9454581|gb|AAF87904.1|AC015447_14 Similar to protein kinases [Arabisopsis thaliana] Length=705	9e-4
B1	~1.2	546	NA	gi|17381028|gb|AAL36326.1| putative arginine methyltransferase pam 1 [Arabisopsis thaliana] Length=390	1e-51
B3	~1.4	496	605	gi|12322128|gb|AAG51102.1|AC025295_10 unknown protein [Arabidopsis thaliana] Length=724	1e-45
B4	~2.0	723	NA	gi|15375406|dbj|BAB63915.1| ERD4 protein [Arabidopsis thaliana] Length=640	2e-76
C1	~1.4	575	NA	gi|22711570|gb|AAN04521.1| Hypothetical protein with similarity to putative retroelements [Oryza sativa (japonica cultivar-group)] Length=237	2e-09

C3	~1.2	724	NA	gi|17381028|gb|AAL36326.1| putative arginine methyltransferase pam 1 [Arabidopsis thaliana] Length=390	3e-65
S1	~1.5	536	642	gi|28564636|dbj|BAC57818.1| PO577B11.15 [Oryza sativa(japonica cultivar-group)] Length=1133	6e-68
S2	~1.2	590	705	gi|23297369|gb|AAN12952.1| arginine methyltransferase pam 1 [Arabidopsis thaliana] Length=390	6e-61

* fragment length is the length as found by restriction digestion in Fig. 3

[1] forward sequence is the number of base-pair in the chromatogram marked and selected for blastx

[2] reverse sequence - same as above

[3] E value or the Expert value is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size

CONCLUSIONS

One of the interesting issues currently in crop biotechnology concerns the minimum components needed to transform a C_3 crop, such as rice, to a more productive C_4 system. The inducible *Hydrilla* system provides an excellent opportunity to study the minimum biochemical elements to operate a C_4 photosynthetic system. Low [CO2] and high temperatures are two environmental parameters involved in the shift of *Hydrilla* from C_3- to C_4-photosynthesis. The unique facultative nature of this plant allows the simultaneous examination of the genes involved in both the C_3 and C_4 states and enables us to document the differences in their expression. In addition, the differential display used here is an excellent method for studying potentially up-regulated genes when the C_4 system is induced, and differences in transcription between the two photosynthetic states.

The C_4 plant material showed up-regulation of at least five genes visible by differential display. It is evident from the these results that a number of up-regulated or uniquely expressed components exist in addition to key enzymes such as PEPC. It is also encouraging that these results were obtained using only four primers, out of potentially 96 (Consalez et al, 1999). This suggests that differential display is a very appropriate method for mining more C_4-specific components from this plant.

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