Journal of Plant Biology, December 1999, 42(4) : 321-324 **PRELIMINARY REPORT**

Studies on Genes and Biochemical Events Induced by Carbon-Source Starvation in Plant Cells

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Carrot (*Daucus carota* L.) suspension cells exhibit a number of physiological responses when carbon sources in the medium are depleted (i.e., carbon-source starvation). We previously reported that activities of several phospholipid catabolic enzymes, such as phospholipase D (PLD) and lipolytic acyl hydrolase (LAH), are induced to provide cells with alternative carbon sources. In this study we report sequence of PLD cDNA. When starvation was prolonged over approximately five days, cells started to die. To analyze the initiation of cell death, we examined the degradation of DNA and activity of DNA endonuclease. Preliminary results showed that DNA degradation occurred at the onset of cell death. Our findings suggest that carrot cells exhibit two different phases--acclimation response and cell death--during starvation. In working toward a long-term objective of understanding the whole scope of biochemical events during starvation, we have also catalogued the genes induced by starvation.

Keywords: carbon starvation, carrot cells, phospholipase D

When carbon sources become limited, plant cells adjust their metabolic strategy as an acclimation response for coping with adverse growing conditions. For example, leaves sacrifice their cellular membranes during senescence to induce phospholipid catabolic pathways; this involves the PLD-LAH-β-oxidation-glyoxylate cycle (Graham et al., 1994; Thompson, 1998). Leaf senescence is accompanied by the absence of photosynthesis and, hence, by the lack of a carbon supply. Therefore, it has been assumed that suspension cells under carbon-source starvation would induce biochemical activities similar to those observed in senescing leaves.

Previously, we reported these senescence-like activities in carrot suspension cells (Lee et al., 1998). We showed that a phospholipid catabolic pathway was induced in a controlled and sequential manner: PLD → LAH → β-oxidation → glyoxylate cycle. PLD is likely the first among those enzymes to be involved in phospholipid degradation. Recently, PLD has received much attention because it was found to be involved in a number of signaling systems in animals and yeast (Billah, 1993; Exton, 1994). In this paper, we report the cDNA sequence of the PLD gene and its expressional pattern.

Those components involved in the phospholipid catabolic pathway remain active up to four days (d 4) after the initiation of starvation. Once starvation extends beyond d 4, these activities sharply decrease and cells start to die. Therefore, we might conclude that once starvation occurs, cells initially acclimate to maintain cellular integrity prior to death. This experimental system should be an excellent way to study how cells initiate death.

One of the hallmarks of this process is DNA degradation, which occurs at the initiation of cell death in animals and plants. A death-specific DNA endonuclease involved in DNA degradation in yeast has been reported by Enari et al. (1998). In their study, the pattern and participation of DNA degradation were examined as a preliminary step to understanding how DNA degradation was induced in these cells under starvation.

We also report the genes induced by starvation as an initial step to describing the biochemical events during cell death. Carrot suspension cells, actively growing with sufficient glucose, were transferred into a glucose-free medium at day zero (d 0) to establish carbon-source starvation. Cells starved for one through to five days (d1-d5) were pooled, then used for establishing a cDNA library to determine the genes expressed in these cells. Total RNAs were prepared from these carrot suspension cells. We isolated polyadenylated mRNA from total RNA with a Poly A tract system (Promega, USA). The cDNA was synthesized from 2 μg of mRNA, using the Universal Riboclone cDNA synthesis system (Promega, USA) with an oligo

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Table 1. DNA sequences identified from the carrot cDNA library. (The detailed procedures are described in the text.)

CLONE	Putative Identification	Organism	Homology (DNA)
CLONE 1	Ribonucleotide reductase M2 subunit	*Melanochromis, auratus*	91%
CLONE 2	Chromosome II BAC T32G6 genomic sequence	*Arabidopsis thaliana*	82%
CLONE 3	Arylmalonate decarboxylase	*Alcaligenes brochisepticus*	97%
CLONE 4	D253 genomic DNA (clone AG13)	*cyanobacterium Calothrix* sp.	87%
CLONE 5	16s rRNA gene, partial sequence	*Shewanella alga*	79%
CLONE 6	NADH dehydrogenase	*Ricinus communis*	83%
CLONE 7	Cytochrome P450 with active lanosterol 14-demethylase gene	*Saccharomyces cerevisiae*	81%
CLONE 8	Hondoense CT repeat DNA	*Rhododendron metternichii*	79%
CLONE 9	BARE-1 long terminal repeat DNA	*Hordeum vulgare*	83%
CLONE 10	16s rRNA gene, partial sequence	*Proteus vulgaris*	81%
CLONE 11	T-DNA integration target DNA	*Oryza sativa*	78%
CLONE 12	mRNA EN320 partial sequence	*Oryza sativa*	69%
CLONE 13	5HT1D gene	*Fugu rubripes*	71%

(dT) primer. It was inserted into a pBluescript vector, then transformed into *Escherichia coli* DH5α cells.

Sixty-three colonies, with an insert length of over 0.3 kb, were used for the random DNA sequencing. We identified 13 different genes from this cDNA library (Table 1). Sequencing from the 5'-end of the cDNA clones was performed with an ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer, USA). Plasmid DNA for the sequencing reaction was prepared by using a QIA prep Spin Miniprep Kit (QIAGEN, Germany). The EST sequences were read automatically and then manually to remove the vector sequences. We performed homology searches using the BLASTX program against the Gen Bank database through NCBI (The National Center for Biotechnology Information).

The genes identified here did not provide an immediate indication of any expected starvation-induced biochemical event. However, these genes may provide some clues once more biochemical or physiological events are understood. For example, one clone highly homologous to the arylmalonate decarboxylase gene of bacterium *Alcarigens brochisepticus* may be used to examine the participation of this particular enzymatic step in carrot cells under starvation. We were not able to obtain the PLD gene by this method.

To clone a PLD gene, we used degenerate primers (from *Ricinus communis* L.) to screen the cDNA library prepared from carrot suspension cells that had been starved one to five days. The expected PCR product (1.0 kb) was subcloned into the pGEM-T vector for further analysis. The cDNA sequence of the putative PLD gene is shown in Figure 1. This sequence is 76% identical to the PLD gene from *R. communis* at the DNA level (Wang et al., 1994). Using this clone, we conducted northern hybridization experiments to examine the pattern of PLD expression in starved carrots cells (data not shown). Here, PLD transcription was active at d 1 and d 2, which agrees with the pattern of PLD activity reported previously (Lee et al., 1998).

As a preliminary step to understanding the initiation of cell death in these carrot cells under starvation, we examined the DNA degradation pattern. Nuclear DNA was prepared daily from cells harvested during starvation. The DNA was incubated for 4 h with the crude extracts prepared daily from the cells. After incubation, aliquots of the DNA were loaded on an agarose gel (Fig. 2). DNA degradation was evident in d-6 cells, which suggests that cells start to die around that time. DNA endonuclease also participated in the degradation.

To quantify the activity of DNA degradation, we adopted a method for measuring the amount of histone released into the cytoplasm (Lyer et al., 1996). At the onset of cell death, nuclear DNA usually is cleaved and chromatin structure becomes destabilized to release histone into the cytosol. Here, a histone antibody was used to detect the level of histone present in the cytoplasm, following manufacturer instructions (Cell Death Detection ELISA, Boehringer-Mannheim, USA). The release became active at d 5 (Fig. 3). This also indicates that DNA endonuclease participates in the degradation of chromatin structure and plays a role in inducing cell death.

This study shows that carrot cells exhibit a transitional phase from acclimation to cell death during carbon-source starvation. Prolonged starvation somehow induces DNA degradation to initiate cell death.

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clone                    30 CATGACGATTTTCATCAACCTAATTTCGAAGGTGCTGCAATTACCANAGGTGGACCTAGG 89
                            ||||| |||||||||||| || || || | |||||| |||||   | ||||| || ||
Ricinus communis       3308 CATGATGATTTTCATCAGCCCAACTTTGCTGGTGCTTCAATTGAAAAAGGTGGTCCAAGA 3367

clone                    90 GAGCCTTGGCATGATATCCACTCNCGCCTTGAAGGTCCAATTGCTTGGGATGTNTTGTTT 149
                            || |||||||||||| |||||||| | ||||||| ||||||||||||||||| ||||||
Ricinus communis       3368 GAACCTTGGCATGACATCCACTCCAGACTTGAAGGACCAATTGCTTGGGATGTTTTGTTT 3427

clone                   150 AATTTTGAGCANAGATGGAAAAAGCNAGGTGGAAAAAATGTACTTGTTAATTTGAGAGAG 209
                            |||||||||||| ||||||| ||||| |||||| ||| |  | ||  || |  |||||||
Ricinus communis       3428 AATTTTGAGCAGAGATGGAGAAAGCAAGGTGGTAAAGACCTGCTCATTCAGCTGAGAGAA 3487

clone                   210 CTTGAGAACATCATCATCCCACCATCAGCAGTGACATTTCCTGATGACNATGACACATGG 269
                            || ||  |  | ||||| || |||||  | || |  | |||||||||| |||  |||||
Ricinus communis       3488 CTAGAAGATGTTATCATTCCCCCATCTCCTGTTATGTATCCTGATGACTTTGAGGCATGG 3547

clone                   270 AATGTTCANTTATTCNNATCCATTGATGGTGGAGCTGCTTTTGGCTTCCCCGACACACCT 329
                            ||||| || || ||  ||||||||||||||||||||| ||||| ||||| || ||||||
Ricinus communis       3548 AATGTCCAGTTGTTTAGATCCATTGATGGTGGAGCTGCATTTGGTTTCCCTGAGACACCT 3607

clone                   330 GAAGCAGCNGCTAAAGCNGGTCTTATCAGTGGAAAAGATAATATAATTGATCGAAGTNTT 389
                            |||| || |  | || || ||| |||||||||| ||||| || |||||  |||||| ||
Ricinus communis       3608 GAAGATGCGCCAGAGGCTGGGCTTGTCAGTGGAAAGGATAAGATCATTGACCGAAGTATT 3667

clone                   390 CANGATGCTTATATTCATGCTATTCGACGGGCAAAAAATTTCNTTTATATCGAAAA1CAG 449
                            || |||||||||| ||||| |||||| ||||||| ||||| ||||||| |||||||||
Ricinus communis       3668 CAGGATGCTTATATCCATGCCATTCGAAGGGCAAAGAATTTTATTTATATTGAAAATCAG 3727

clone                   450 TATTTTCTCGGAAGTTCTTTTGGCTGGAACTCACAGGATATAATTGATGCAGATGTCAAT 509
                            ||||| || |||||||||||||| ||||  |  | | ||| |   ||  ||| | |||
Ricinus communis       3728 TATTTCCTTGGAAGTTCTTTTGGTTGGAGTCCTGATGGTATTAAGCCTGAGGATATTAAT 3787

clone                   510 GCTTTGCATCTAATACCNAAGGAGCNCTCCCTGAAGATTGTCNGTAAAATCGAANCTGGA 569
                            ||  |||||||||||| ||||| |  || || ||||| |  | || || |  | ||
Ricinus communis       3788 GCACTGCATCTAATACCCAAGGAACTTTCACTCAAGATACTTAGCAAGATTGCGGCAGGG 3847

clone                   570 GANAAGTTCCTNGTT 584
                            || | ||||   |||
Ricinus communis       3848 GAGAGGTTCACTGTT 3862
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Figure 1. The cDNA sequence of phospholipase D from carrot and its homology with that from *R. communis*. The cDNA sequence from carrot is shown in the upper line, and the sequences identical with those from *R. communis* are vertically aligned.

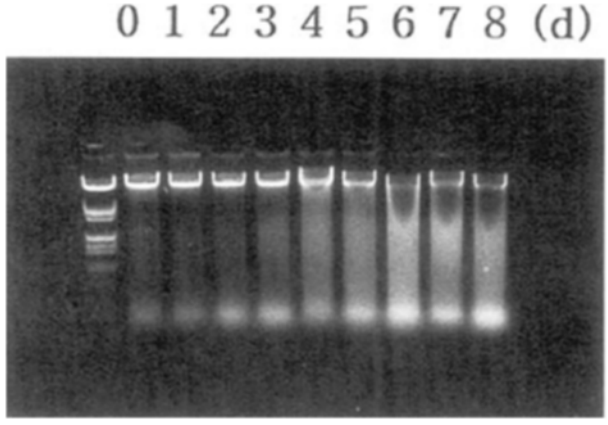

Figure 2. DNA degradation in cells under carbon-source starvation. Two μg of isolated nuclear DNA from actively growing cells were digested in the presence of extracts prepared from cells harvested daily after the initiation of starvation. Following the digestion, the DNA was separated on a 0.8% agarose gel and stained with ethidium bromide. The significant degradation of DNA is evident in d-6 cells. The numbers indicate days after starvation.

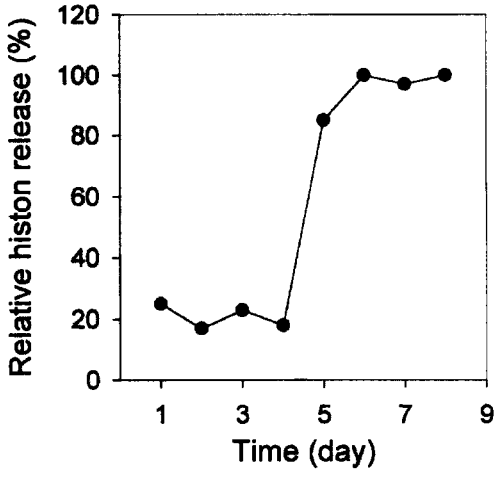

Figure 3. Measurement of DNA endonuclease-mediated DNA degradation by detection of histone released into cytosol. Cells were harvested daily after the initiation of starvation. Detailed procedures were described in the text.

Our long-term objective is to understand how cells recognize the time point at which to initiate death. Presently, we are investigating several probable signal elements, such as active oxygen, which also participates in the precipitation of cell death.

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