Journal of Plant Biology, April 2006, 49(2) : 133-140

Identification of Differentially Expressed Genes Involved in Spine Formation on Seeds of *Daucus carota* L. (Carrot), Using Annealing Control Primer (ACP) System

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Carrot seeds normally have surface spines. The availability of a spineless mutant would be agronomically beneficial, eliminating the current efforts to remove those spines pre-sowing. Furthermore, the identification of spine-specific genes would provide insights into spine development in wild-type carrot seed. This effort could be facilitated through the use of an annealing control primer (ACP) system. Here, we employed a new and accurate reverse transcription-polymerase chain reaction (RT-PCR) that involves ACPs for identifying genes of interest. With these techniques, 11 expressed sequence tags (ESTs) were obtained for cloning and sequencing the genes that are differentially expressed in wild-type spiny seeds, but not in the spineless mutant. In all, 7 cDNAs exhibited significant sequence similarity with known genes from other species. These included cell wall-associated hydrolase, tail fiber assembly protein, transcriptional regulatory protein, berberine bridge enzyme, S-adenosyl methionine synthase, transketolase, and phenylalanyl t-RNA synthetase beta chain. Four other cDNA sequences had no significant identities with known genes. As revealed by RT-PCR, these genes regulate spine formation during the developmental stage. Our results suggest that PCR-based differential display RT-PCR techniques are a very useful tool for identifying spine-specific genes from carrot seeds.

Keywords: annealing control primer (ACP), carrot, differentially expressed gene (DEG), RT-PCR, spineless, spiny

Carrot (*Daucus carota* L.) seeds normally have spines on their testa. However, a spineless mutant was serendipitously found in 1998 by Dr. Yong Park while developing maintainers for the carrot cytoplasmic-genetic male sterile (CGMS) system at the Jung Ang Seed Company in Korea (Fig. 1). This character would be very beneficial to the agronomic industry because, currently, the spines must be removed prior to sowing. Moreover, in addition to this being a laborious effort, the viability of those seeds can be reduced by such mechanical force (unpublished data). Segregation ratios for normal and mutant plants are approximately 1:1 and 3:1 in the maintainer and male sterile lines, respectively, suggesting that this mutant is controlled by a recessive gene (Park, 1998). Inheritance of the spineless character is apparently not controlled by a single recessive allele because the selfed progenies of a spineless plant among those maintainer lines can be segregated for spiny, spineless, and semi-spineless genotypes (Park et al., 2002).

The seed spine (seed hair) is a differentiated, single epidermal cell of the seed coat. Light microscopy of spineless seeds has revealed that the division and/or elongation of cells that eventually develop into spines seem to be stopped at a very early stage of seed formation (Park et al., 2002). Spiny seeds have multicellular appendages that originate from the subepidermal layer. Other than those characteristics, no differences in numerous agronomic traits can be observed between spineless and spiny lines (Park et al., 2002).

Our knowledge of spine formation in carrot seed is limited, as is any characterization of the proteins involved in spine development, cell wall formation, cellulose synthesis, and cytoskeletal assembly. To further understand the molecular basis for spine production, researchers must identify and perform detailed profiling of novel, differentially expressed genes (DEGs), and analyze their functioning. To this purpose, several reverse transcription-polymerase chain reaction (RT-PCR) technologies have been applied.

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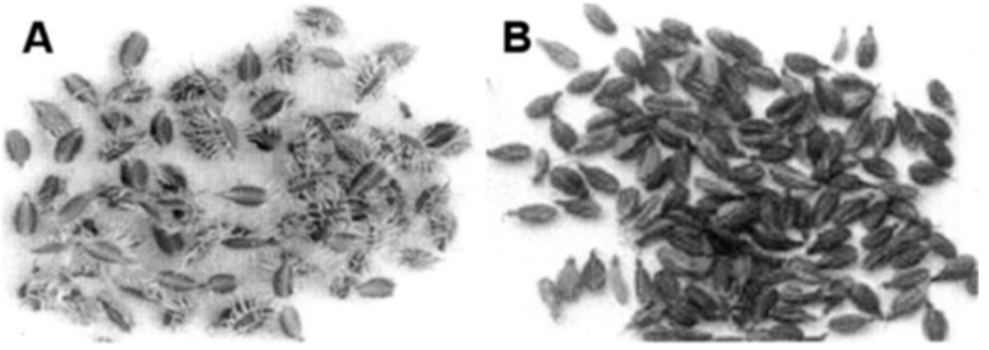

Figure 1. Spiny (**A**) and spineless (**B**) carrot seeds.

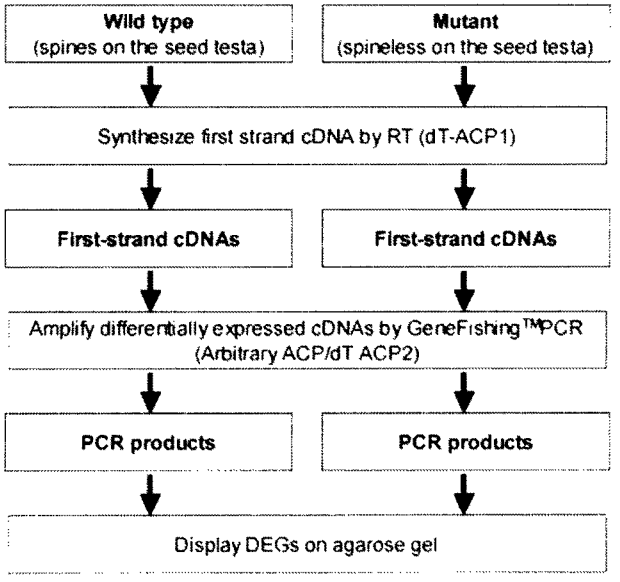

Figure 2. Schematic depiction of ACP RT-PCR GeneFishing procedure. mRNAs isolated from wild-type (spines on seed testa) and mutant (spineless) carrot seeds were used for synthesis of first-strand cDNA with dT-ACP1 primer. Second-strand cDNAs were then amplified during second-stage PCR with a combination of dT-ACP2 (reverse primer) and 1 of 120 arbitrary ACP primers (forward primer). Products were separated on agarose gel to identify DEGs showing greater expression in the wild type.

However, in the study described here, we used a more accurate and extensive PCR technology, which was controlled by an annealing control primer (ACP) that specifically targets sequence hybridization to the template via a polydeoxyinosine [poly (dI)] linker. This method (Fig. 2) has been applied to analyze DEGs specifically in the spine (Hwang et al., 2003). Our primary objectives were to investigate the molecular mechanism(s) involved in spine development and to compare the expression patterns for genes from seeds of wild-type and mutant carrot lines.

MATERIALS AND METHODS

Plant Material

Carrot (*Daucus carota* L.) plants were grown at the Daegu University Experimental Farm in Gyungsan City, Korea. Spineless-seed mutants had been, by chance, obtained when petaloid types 95012 and 95013 were crossed (Park et al., 2002). Fertilized flowers containing immature seeds in their ovaries were plucked and immediately frozen in liquid nitrogen for storage at -80°C.

First-Strand cDNA Synthesis

Total RNAs extracted from carrot umbels were used for the synthesis of first-strand cDNAs (Choi et al., 2002; Oh et al., 2004). Reverse transcription was performed for 1.5 h at 42°C in a final reaction volume (20 μL) containing 3 μg of the purified total RNA, 4 μL of 5′ reaction buffer (Promega, USA), 5 μL of dNTPs (2 mM each), 2 μL of 10 μM dT-ACP1 (5′-CGTGAATGCTGCGACTACGATIIIIIT(18)-3′), 0.5 μL of RNase Inhibitor (40 U/μL; Promega, USA), and 1 μL of Moloney murine leukemia virus reverse transcriptase (200 U/μL; Promega). First-strand cDNAs were diluted by the addition of 80 μL of ultra-purified water for GeneFishing™ PCR, and then stored at -20°C.

ACP-Based GeneFishing™ PCR

Differentially expressed genes were screened by the ACP-based PCR method (Kim et al., 2004) using the GeneFishing™ DEG kit (Seegene, Korea). Briefly, second-strand cDNA synthesis was conducted at 50°C during one cycle of first-stage PCR, in a final reaction volume of 20 μL containing 3 to 5 μL (about 50 ng) of diluted first-strand cDNA, 1 μL of dT-ACP2 (10 μM), 1 μL of 10 μM arbitrary ACP (Table 1), and 10 μL of

Table 1. Primer sequences used in cDNA synthesis and ACP-based[a] PCR.

Primer type	Primer name	Sequence[b]
	dT-ACP1	5′-CTGTGAATGCTGCGACTACGATIIIII$(T)_{18}$-3′
	dT-ACP2	5′-CTGTGAATGCTGCGACTACGATIIIII$(T)_{15}$-3′
	ACP1	5′-GTCTACCAGGCATTCGCTTCATIIIIIGCCATCGACC-3′
	ACP15	5′-GTCTACCAGGCATTCGCTTCATIIIIICCACCGTGTG-3′
	ACP43	5′-GTCTACCAGGCATTCGCTTCATIIIIIAGAAGCCGTC-3′
cDNA synthesis primer	ACP48	5′-GTCTACCAGGCATTCGCTTCATIIIIIAGAAGCCTGC-3′
Reverse primer	ACP57	5′-GTCTACCAGGCATTCGCTTCATIIIIIAGCCGACCTC-3′
Arbitrary primer (forward primer)	ACP58	5′-GTCTACCAGGCATTCGCTTCATIIIIIATAAGATTGT-3′
	ACP68	5′-GTCTACCAGGCATTCGCTTCATIIIIIACCGACTGGT-3′
	ACP77	5′-GTCTACCAGGCATTCGCTTCATIIIIICTCCGATGCC-3′
	ACP87	5′-GTCTACCAGGCATTCGCTTCATIIIII-GGCAACGGCG3′
	ACP101	5′-GTCTACCAGGCATTCGCTTCATIIIIICCCTGGAACT-3′
	ACP115	5′-GTCTACCAGGCATTCGCTTCATIIIIIATACGGGCCC-3′

[a], ACP, annealing control primer.
[b],The polydeoxyinosine [poly(dI)] linkers are underlined. I represents deoxyinosine.

2′ Master Mix (Seegene). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s; followed by 65°C for 40 s and 72°C for 40 s; then a 5-min final extension at 72°C. The amplified PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

Cloning and Analysis of cDNA

Differentially expressed bands were extracted and cloned into a pGEM T-Easy vector (Promega, USA) following the manufacturer's instructions. The ligation mixture was transformed into competent *Escherichia coli* DH5α cells; plated onto LB agar containing 100 μg mL ^{1} ampicillin, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 80 μg mL^{-1} X-gal; and incubated at 37°C overnight. White colonies that contained inserts were then grown overnight on LB liquid media supplemented with 100 μg mL^{-1} ampicillin. Plasmid DNA was isolated from randomly selected, white colonies by a commercial kit (Invitrogen, USA). To verify the identity of insert DNA, isolated plasmids were sequenced automatically. DNA sequencing was performed by dye-terminator cycle sequencing using the BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystem, USA), followed by ethanol precipitation of the extension products. Both procedures were performed according to the manufacturer's instructions. The T7 and SP6 primers were used to generate sequences for all isolated cDNAs. Cycle sequencing was performed in a GeneAmp PCR System 9800 Thermal Cycler (Perkin-Elmer Cetus, USA). Sequences were edited manually to remove contaminants originating from the vector or primer sequences and those corresponding to polyA tails, as well as to discard poor-quality sequences. To assign functions to the cluster consensus of the remaining cDNAs, the derived amino acid sequences were compared with amino acid sequences deposited in public databases, using the BLASTX search algorithm (http://www.ch.embnet.org/software/BottomBLAST.html). Functional categories were based on those used for annotation of the *Arabidopsis thaliana* genome (The Arabidopsis Genome Initiative, 2000).

RT-PCR Quantification

To validate the results of ACP differential display and to determine the relative abundance of target sequences, RT-PCR was performed using total RNA isolated from wild-type and mutant seeds. RT-PCR analysis also was undertaken of the expression patterns for individual genes specific to either genotype. mRNA expression patterns of cell wall-associated hydrolase, tail fiber assembly protein, manganese superoxide dismutase, S-adenosyl methionine synthase, transketolase, and phenylalanyl-tRNA synthetase beta chain, plus five unknown genes were used for RT-PCR with All-in-one RT/PCR Premix (P7003; SuperBio, Korea). Amplification of 60S ribosomal RNA served as an internal control. Prior to RT-PCR amplification, sequence-specific primers were designed from DEGs cloned from the ACP differential display, using the QuickChange primer TM calculator (http://www.artsci.wustl.edu/~twest/molbio/qctemp.php). For optimal quantification, parameters were set

Table 2. Identity and size of carrot DEGs that encode unknown proteins, and their homology to sequences in GenBank.

	Identity	Clone #	% Sequence identity (no. of a.a.)	GenBank No.	Species sharing homology
I	Cell wall-associated hydrolase	GP68	73% (90 a.a)	Q8CME1	*Vibrio vulnificus*
	Tail fiber assembly protein	GP87	33% (42 a.a)	Q72C01	*Desulfovibrio vulgaris*
	Transcriptional regulatory protein	GP101	53% (26 a.a)	Q9COZ1	*Schizosacharomyces pombe*
II	Berberine bridge enzyme	GP48	50% (81 a.a)	Q570D1	*Arabidopsis thaliana*
	S-adenosyl methionine synthase	GP57	97% (42 a.a)	Q94FA6	*Brassica juncea*
	Transketolase	GP58	84% (93 a.a)	O20250	*Spinacia oleracea*
	Phenylalanyl-tRNA synthetase beta chain	GP115	33% (66 a.a)	Q633N5	*Bacillus cereus*
III	Unknown	GP1			
	Unknown	GP15			
	Unknown	GP43			
	Unknown	GP77			

to design primer sequences with a melting temperature (Tm) of 60°C and a product size of 150 to 350 bp (Table 2). Total RNA (100 ng) was used for RT-PCR, in a volume of 20 μL containing 20 mM Tris-HCl (pH 7.9), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, M-MLV reverse transcriptase (RNaseH Minus), Super Taq plus DNA polymerase, and 10 pmol of each gene-specific amplification primer (Table 1). The procedure was conducted at 50°C for 30 min, then inactivated at 96°C for 3 min. PCR amplification was performed in a GeneAmp PCR system 9700 Cycler (Perkin-Elmer, USA), with 25 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min; followed by a final extension at 72°C for 10 min. The resultant RT-PCR products were resolved on a 1% agarose gel, stained with ethidium bromide, and photographed.

RESULTS

mRNA Expression Patterns

To identify and isolate the DEGs from wild-type carrot seed, we compared their mRNA expression profiles with those from the spineless mutant. Total RNA isolated from fertilized flowers that contained immature seeds was subjected to the ACP system. For this, total RNA isolated from both genotypes was used as template for first-strand cDNA synthesis, with dT-ACP1 as the primer (Table 1). Then, with first-strand cDNA as template, second-strand cDNAs were synthesized during one cycle of first-stage PCR that utilized arbitrary ACP primers. Through the combination of dT-ACP2 (reverse primer) and 120 arbitrary ACPs (forward primer), second-strand cDNAs were then amplified during second-stage PCR at a second annealing temperature (65°C), which constituted high-stringency conditions. Sequences at the 3'- and 5'-ends of the second-strand cDNAs served as templates for this amplification. On the basis of differential expression levels for the mRNA fragments observed on the agarose gels, and using 11 arbitrary ACPs, 11 bands were detected from the wild type (spiny) but not from the spineless mutant (Fig. 3). These were excised from the gels, cloned, and sequenced for analysis. Expression patterns for these cloned DEGs - GP1, 15, 43, 48, 57, 58, 68, 77, 87, 101, and 115 - were then confirmed by RT-PCR (Fig. 4). The RT-PCR assays revealed that, consistent with the ACP differential display, transcripts of the genes had similar expression patterns when DEGs from the wild type were compared with those of the mutant. In addition, four DEGs - GP48-2, GP71, GP101-2, and GP113 - were over-expressed specifically in the mutant (Fig. 4).

Functional roles, sequence similarity, and characterization of differentially expressed transcripts are summarized in Table 2. BLASTX searches in the NCBI GenBank revealed that seven DEGs (GP48, 57, 58, 68, 87, 101, 105) showed significant similarity with known genes, while four others (GP1, 15, 43, 77) did not significantly match any known proteins in the public databases. All genes or ESTs identified and characterized in this study have already been submitted to GenBank and assigned accession numbers (Table 3).

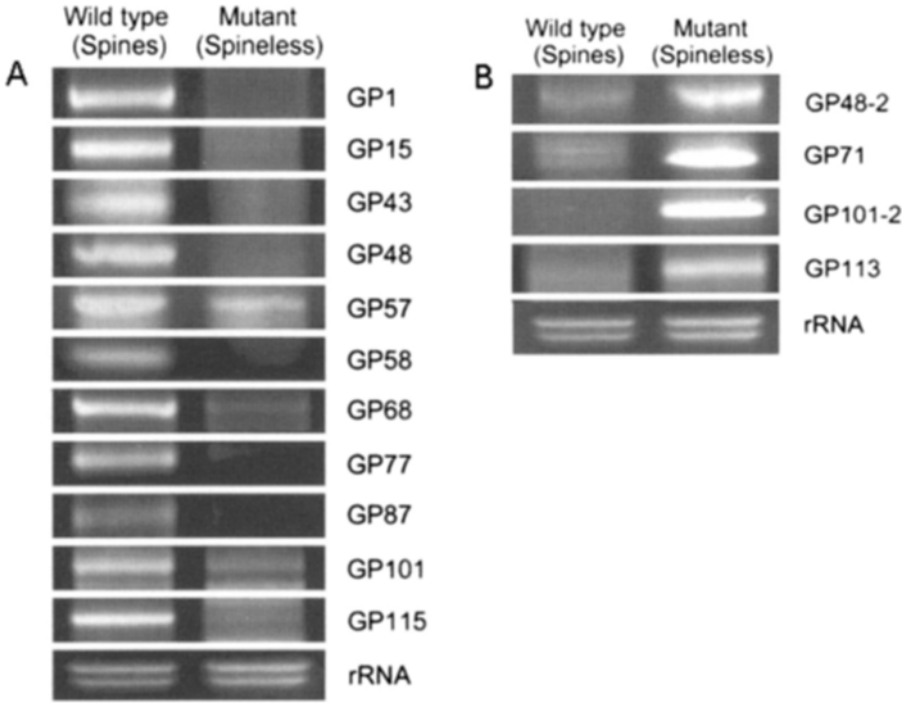

Figure 3. Differential expression of genes/sequences between two carrot genotypes (with and without spines). Results of ACP-based PCR for identification of differentially expressed genes (DEGs) for wild-type (spines on seed testa) (**A**) and mutant (spineless) (**B**). Gels show differential banding patterns obtained from two different samples produced *in vitro* using a set of arbitrary ACP (5′-primer) and dT-ACP2 (3′-primer). Amplified cDNA products were separated on 2% agarose gels and stained with ethidium bromide. cDNA bands were excised from gel for further cloning and sequencing.

Table 3. Sequence-specific primers used for quantification of differentially expressed transcripts.

Clone name	Primer name	Sequence	GenBank No.	Product length (bp)
GP1	*GP1-S*	TCGCAGTTCTAGTATCTTATG	DY306892	150
	GP1-AS	GGTGCCCATAGATATAAAATAAA		
GP15	*GP15-S*	GCAGTTCCCCAACACAG	DY306891	264
	GP15-AS	CCTCATCACTTGAACTGGA		
GP43	*GP43-S*	AAGACTCCAGCTGATCTTCA	DY306897	155
	GP43-AS	TTCTCGACTCTCAAACAGGT		
GP48	*GP48-S*	GTCATTGGAAAGACTCCAG	DY306896	181
	GP48-AS	GGAAGCGAACGATTGGTAT		
GP57	*GP57-S*	GAAGACCATCTTTCACCTCA	DY306895	302
	GP57-AS	TTTCCAGTTCCATAGGTGTC		
GP58	*GP58-S*	CGTGTGCGAACTTTAATTTTAC	DY306894	155
	GP58-AS	CCGAAGATATGATAAATAACAGAA		
GP68	*GP68-S*	CTGGTCGAACAGAGACG	DY306893	263
	GP68-AS	ACGGCAGATAGGAACCG		
GP77	*GP77-S*	GCCAGCGCAAAGGCATA	DY306890	246
	GP77-AS	GATTGAACTCTCTTGCTACAT		
GP87	*GP87-1-S*	GCGCATCAGCACTCGTT	DY306889	264
	GP87-1-AS	TTGCAAACTGATTTTATTATTGGTA		
GP101	*GP101-S*	GCATACAGAAATTTAAATCCCAA	DY306899	210
	GP101-AS	TGTATGTTTGTTTCCTTTGGATT		
GP115	*GP115-1-S*	GCATCAGCACTCGTTTTAG	DY306888	262
	GP115-1-AS	TTGCAAACTGATTTTATTATTATTGGTA		
40S Ribosome	*Ribo-S*	CTGGTCTTGAACCAACTGA	DY306898	350
	Ribo-AS	CAAGCATAGCATCATGAACA		

Nucleotide Sequencing and Homology Searches

Using ACP-based differential display, 11 spine-specific cDNA clones were isolated from normal carrot seeds. The information garnered from sequence analysis was evaluated by comparing it via BLASTX with conceptual partial amino acid sequences deposited in public databases. The cDNA sequences were then compared with GenBank databases to identify possible homologies. Here, the GP48, 57, 58, 68, 87, 101, and 115 sequences were 50, 97, 84, 73, 33, 53, and 33% identical to the product for S-adenosyl methionine synthase cDNA, S-adenosyl methionine synthase, transketolase, cell wall-associated hydrolase, tail fiber assembly protein, transcription regulatory protein, phenylalanyl-tRNA synthetase beta chain, respectively (Table 3). Based on these results, three groups of clones were established: putative cell wall proteins, stress-inducible enzymes, and cDNA clones with no homology (Table 2).

Group I: Putative Cell Wall Proteins

cDNA clones in Group I shared homology with a gene that encodes a putative cell wall protein isolated from *D. carota*, which has normal spine formation on its seed coat. In this group, GP68, GP87, and GP101 gave highly significant sequence matches with cell wall-associated hydrolase, tail fiber assembly protein, and transcriptional regulatory protein, respectively (Table 2). Although the cDNAs clones of Group I were expressed exclusively in normal carrot seeds, this was not yet conclusive enough to relate these clones to spine formation.

Group II: Stress-Inducible Enzymes

Group II comprised cDNA clones with similarities to genes encoding metabolic enzymes. Here, GP48, GP57, GP58 and GP115 gave highly significant sequence matches with berberine bridge enzyme, S-adenosyl methionine synthase, transketolase, and phenylalanyl-tRNA synthetase beta chain, respectively (Table 2). These four genes are reportedly induced by wounding or pathogen, and may possibly be involved in secondary metabolite biosynthesis.

Group III: DNA Clones with No Known Homologies

Four cDNA clones - GP1, GP15, GP43, and GP77 - were included in Group III, which contains genes with no significant homologies, based on searches of nucleotide and protein databases using both BLASTN and BLASTX programs.

Confirmation of ACP Observations by RT-PCR

To confirm the efficacy of the ACP system and to further investigate wild type-specific expression of the genes identified here, we used RT-PCR to analyze the patterns of 11 genes selected from a comparison between the wild type and the mutant. Sequence-specific primers were designed to amplify at lengths of 150 to 350 bp (Table 2). To normalize the efficiency of the RT-PCR reaction, a ribosome gene was used as an internal standard. When expression of the wild type (spines on the seed testa) was compared with that of the mutant (spineless), all of our target transcripts showed expression patterns in agreement with the results of the ACP differential display (Fig. 4). Therefore, we propose that these genes are involved in spine formation, based on the up-regulation patterns observed for the wild type. Our results indicate that the genes are coordinately expressed during spine formation, and that their levels of expression seem to be positively related to that development.

Discussion

To study the mechanism for spine formation on carrot seed testa, and to identify the genes specifically or predominantly expressed in the wild type, we employed a new differential display RT-PCR technique (Hwang et al., 2003; Kim et al., 2004; Cui et al., 2005). Expression patterns were compared between the spiny wild type and the mutant. This more accurate system lacks false positives and utilizes extensive PCR technology controlled by an ACP. The annealing control primer has a unique tripartite structure consisting of 3′- and 5′-end distinct portions separated by a regulator. This ACP structure comprises a 3′-end region with a target core nucleotide sequence that substantially complements the template nucleic acid for hybridization, plus a 5′-end region with a non-target universal nucleotide sequence, and a poly(dI) regulator region bridging the 3′- and 5′-end sequences (Hwang et al., 2004). The ACP linker prevents annealing of the 5′-end non-target universal sequence. This ACP-based PCR system facilitates the identification of DEGs from samples with low levels of mRNA (Hwang et al., 2003). The specificity and sensitivity with which a primer anneals to its target sequence are the most critical factors in determining the success of PCR amplification.

Using an ACP system, DEGs have been identified in bovine blastocysts and hatched blastocysts produced *in vitro* (Hwang et al., 2004) as well as in murine embryos at the blastocyt stage (Cui et al., 2005). Maternal mRNAs in porcine parthenotes at the 2-cell stage have also been identified and compared with the blastocyst stage (Hwang et al., 2005). Here, we applied this system for analyzing genes that were differentially expressed between wild-type and mutant seeds. Using dT-ACP2 (reverse primer) and 120 arbitrary ACPs (forward primer) for PCR amplification, we were able to display the differentially expressed cDNA bands specifically in the wild type. Moreover, we identified 11 DEGs that were specifically or more prominently expressed in the wild type than in the spineless mutant. Results of our ACP-based GeneFishing PCR analysis demonstrated that this method is suitable for the isolation of cDNA molecules for genes that are preferentially expressed in wild-type, spiny carrot seeds.

Data from the sequence analyses of the differentially expressed cDNAs revealed that seven DEGs exhibited significantly higher sequence homology with the coding regions of known genes in GenBank (Table 2). For example, GP87 shares similarity with the tail fiber assembly protein, and is abundantly expressed in wild-type seed. Cytoskeleton assembly plays an important role in determining the cell length and morphology of cotton fibers (Kim and Triplett, 2004). However, it is unclear how that assembly is regulated. In the case of GP101 (transcriptional regulatory protein), a complex of regulatory networks controls the close relationship between secondary metabolism and epidermal and seed differentiation in *Arabidopsis* (Broun, 2005). In the current study, both cell wall-associated hydrolase and tail fiber assembly protein had significantly greater expression in the wild type than in the spineless mutant.

Interestingly, stress-inducible enzymes were accumulated at high levels in the wild-type seed. Both S-adenosyl methionine synthase (GP57) and berberine bridge enzyme (GP48) are expressed similarly to phenylpropanoid metabolism (Harding et al., 2005). For example, berberine bridge enzyme is induced in cultured opium poppy cells in response to wounding or treatment with a pathogen-derived elicitor (Park et al., 1999), while transcription of SAMS genes is induced in elicited cells of alfalfa (Gowri et al., 1991). Increased accumulation of *SAMS* transcripts or protein has been repeatedly observed in tissues where lignification occurs (Whetten et al., 2001). Finally, coordinated accumulation of SAMS and METS (Met synthases) occurs during seed germination in *Arabidopsis* (Gallardo et al., 2002).

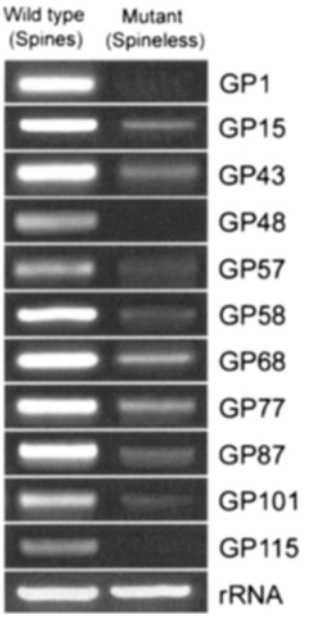

Figure 4. Confirmation by semi-quantitative RT-PCR of differential mRNA expression patterns for 11 genes identified as DEGs by ACP RT-PCR. Comparison of expression patterns for 11 DEGs in wild type and mutant by semi-quantitative RT-PCR. Amplified DNA products were separated on 1.2% agarose gel and stained with ethidium bromide. Ribosome gene served as control to confirm integrity of mRNA samples.

Here, we observed that five unknown genes also were highly expressed in the wild type; quantitative RT-PCR was employed for a more detailed assessment of their expression between wild type and mutant. The level of expression was normalized relative to the ribosome mRNA to reveal changes between the two genotypes (Fig. 4). As expected, 11 genes, including the 5 unknowns, were expressed differentially in the spiny seed. Another 4 were overexpressed specifically in the spineless mutant. Those 4 might be transcription factors that repress the structural genes related to spine formation on the seed testa.

In conclusion, the data presented in this study provide the first global overview of a set of genes in the carrot genome that are expressed in wild-type seeds with spiny testa. Although our results are based only on a subset of genes and, therefore, cannot be considered fully representative, they indicate that many transcriptionally regulated genes are accumulated differentially in wild-type carrot seeds. To date, very little has been known about the mechanism for spine for-

mation. Nevertheless, the approach adopted here is a crucial step in elucidating this anatomical development, and allows the isolation of genes closely associated with it. Further studies are required concerning the biological and biochemical activities of the gene products.

ACKNOWLEDGMENTS

This work was supported by a Research Center for Processing and Application of Agricultural Products (Project No. 20036630), an RRC program of MCIE, and by a basic research fund of Rural Development Administration, Korea.

Received December 20, 2005; accepted February 27, 2006.

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