Cloning and expression of *DnaJ* homolog in carrot somatic embryo

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Received October 9, 2002; revised November 12, 2002

Abstract As the co-chaperone of DnaK/Hsp70 protein, DnaJ/Hsp40 protein influences the synthesis and assembly of the protein complex by regulating ATPase activity of DnaK/Hsp70 protein. By employing the modified method of cDNA representational difference analysis, a homologous fragment of DnaJ was isolated from the deregulated carrot somatic embryos, and it was further used as the probe to screen the cDNA library of carrot somatic embryo deregulated for 12 h. As the result, *DcJ1* gene, the homologous gene of DnaJ, was isolated from carrot. Sequence analysis showed that its coding region is 1257 bp, which codes 418 amino acids and comprises 3 highly-conserved characteristic domains. Southern blot analysis suggested that the *DcJ1* gene seems to be a single copy in the genome, while Northern blot result indicated that *DcJ1* expresses only in roots and its degree of expression changes obviously with the regulation-deregulation process. These results suggest that *DcJ1* is correlated with the early development of carrot somatic embryo radicle.

Keywords: *DnaJ*, carrot somatic embryo, express regulation, heat shock protein.

*DNA* is a heat shock protein that functions together with *DNA* as a molecular chaperone in *Escherichia coli* by stimulating ATPase activity of *DNA* by a normal family of eukaryota in terms of sequence and function, which is essential to many kinds of cell processes. Both *DNA* and *Hsp40* express constitutively under normal conditions, and play roles in folding and import of nascent peptides, assembly of protein complexes, etc. Induced substantially by stresses, such as heat, cold, drought, or salinity, they are also responsible for stabilizing the structures of proteins and denaturing denatured proteins.

During their research on artificial seeds, Huang et al. found that the change of sucrose concentration in MS medium affected the development of carrot somatic embryos, and they developed a regulation-deregulation culture system: under normal circumstances, sucrose concentration in MS medium was 3%, in which carrot somatic embryos developed into young seedlings immediately after embryogenesis and morphogenesis; when sucrose concentration was increased to 5% (regulation culture), the development of somatic embryo was arrested at the stage of cotyledon embryos and, above all, embryo radicle elongation was suppressed; but when these regulated cotyledon embryos were transferred into MS medium containing 1% sucrose, suppression of embryo radicle elongation was released, and somatic embryos developed into plantlets rapidly. The establishment of this culture system can provide a set of perfect materials for the research of molecular mechanism on the development of carrot somatic embryo radicle.

By using the modified cDNA representational difference analysis (cDNA RDA) technique, we isolated gene fragments associated with the development of carrot somatic embryo radicle using this regulation-deregulation culture system, and screened the cDNA library of carrot somatic embryos deregulated for 12 h by these gene fragments as probes. A *DNA* homologous gene, *DcJ1*, from carrot was obtained. Sequence analysis suggested that *DcJ1* is located in the cytoplasm, and comprises 3 highly-conserved domains. And we proved that *DcJ1* expresses only in roots, and its expression is related to the growth and development of embryo radicle. Here, we report our experimental results.

1 Materials and methods

1.1 Plant materials

Carrot (*Daucus carota* L.) used here was a commercial Japanese variety. Carrot somatic embryos were prepared mainly according to the procedure reported by Huang et al. with some modifications. The callus was inoculated in the regulation medium...
directly, in which both the induction and regulation of somatic embryos were accomplished. In the regulation medium, the development of somatic embryo stays at the cotyledon stage. Regulated cotyledon em-
byros were then transferred into the deregulation medium, and then they were harvested for the following ex-
periments after a certain period of further culture.

1.2 Construction and screening of cDNA library

Plant materials were carrot somatic embryos deregulated for 12 h. mRNA extraction and cDNA synthesis were carried out by using QuickPrep™ Mi-
cro mRNA purification kit and TimeSaver™ cDNA synthesis kit from Pharmacia Inc. according to protocols provided by the manufacturer. cDNAs were lig-
ated into Lambda gt10 vector (Promega Inc.). The Packagene Lambda DNA system (Promega Inc.) was used to pack the product of ligation. Titer of the li-
brary was 1 × 10⁶ pfu.

Differential fragment NR3 (321 bp) from RDA, which was labeled with (α³²P) dCTP (specific activ-
ity 3000 × 3.7 × 10¹² Bq/mmol, Ya Hui Inc.) by the Primer-a-Gene® Labeling System (Promega Inc.),
was used as the probe for screening cDNA library mentioned above. Hybridization was performed in a solution containing 6 × SSPE, 5 × Denhardt, 0.5 %
SDS and 100 μg/mL salmon sperm DNA. The mem-
brane was washed 3 times with 2 × SSC, 1% SDS for 30 min. Both hybridization and membrane washing temperature was at 65°C. The autoradiography was performed at −20°C.

1.3 Subcloning and sequencing of target fragments

DNA of positive phage was extracted and digest-
ed with NotI. Restriction fragments of the cDNA in-
sert were subcloned into pBluescript SK-vector (Stratagene Inc.). Recombinants were transformed into E. coli XL1-Blue and spread on LB (Amp⁺, Tet⁻) solid medium on which positive clones were id-
entified by IPTG-X-gal. The positive clones identi-
fied by restriction enzyme digestion were sequenced by Bioasia Corp. The nucleotide and deduced amino acid sequences were compared with those in the Gen-
Bank and EMBL databases by using the sequence analysis program, Genetyx.

1.4 Southern blotting

Genomic DNA from carrot leaves were digested with restriction endonucleases AccI, EcoRI, XbaI, 
BglII and EcoRV, and 10 μg of digested genomic DNA from each group were separated using 1% a-
garose gel electrophoresis and transferred to Nylon membrane (Millipore Corp.). The probe was a 1390 bp cDNA fragment comprising the intact cDNA coding region acquired by digesting DeI clone with EcoRV and NotI. Labeling and hybridization methods were the same as mentioned before. Membrane was washed with a solution of 2 × SSC containing 0.1%SDS at 65°C for 30 min, and repeated 3 times; then washed with 1 × SSC, 0.1% SDS at 65°C for 30 min, and repeated once; and finally with 0.1 × SSC, 0.1% SDS at 65°C for 30 min.

1.5 Northern blotting

Total RNAs were extracted from several groups of samples; leaves, petioles and roots of adult plants; carrot somatic embryos under regulated and dereg-
ulated states for 12 h, 24 h, 36 h and 48 h; and carrot somatic embryos which were heat shocked at 37°C for different periods of time. RNA(10 μg) taken from each group was separated on formaldehyde agarose gels and blotted onto Nylon membrane (Millipore). The probe for RNA gel blot analyses and the conditions for hybridization and membrane washing were the same as in Southern blot analysis.

2 Results and discussions

2.1 Improvement of regulation-deregulation culture system of carrot somatic embryos

In the original regulation-deregulation system of carrot somatic embryos established by Huang et al. the callus was inoculated into hormone-free MS liquid medium containing 3% sucrose. As soon as the global embryos emerged, the cultured material should be transferred to the regulation medium (hormone-free MS liquid medium containing 7% sucrose) immediately. This method needs a person who has a lot of experience and even so, some somatic embryos could still not be regulated for not handling properly. In or-
der to get the regulated somatic embryos with a high quality, we modified the original culture method by increasing the concentration of sucrose in the medium. We found that high sucrose concentration does not suppress induction and genesis of embryos, and effectively controls the germination of embryo rad-
icle. Fig.1 shows the regulated embryos acquired by using our improved culture method for carrot somatic
embryos and the seedlings deregulated for 3 days, in which embryo radicles of all regulated embryos do not elongate no matter what the size of the embryo is. We also observed that after deregulation the development of roots started in all of the embryos. This means that the development status of the embryos we got is consistent.

Fig. 1. Regulated embryos acquired by using improved regulation deregulation culture method for carrot somatic embryos (left) and the seedlings deregulated for 3 days (right).

2.2 Isolation and sequence analysis of homologous gene of DnaJ from carrot

Plant DnaJ gene was first isolated from A. tríplex nummularia\(^7\), and its homologs have been cloned from more than 10 species so far\(^{5-18}\). With the modified cDNA RDA technique we obtained DnaJ homologs from carrot somatic embryos directly. In this method, the cDNA of carrot somatic embryos under regulation was used as a tester and the cDNA from carrot somatic embryos after deregulation for 12 h was used as a driver, and vice versa. Through 3 cycles of subtraction hybridizations, a weak smear appeared in the regulated materials, and 4 cDNA bands designated NR1, NR2, NR3 and NR4 occurred in the deregulated materials. After sequencing and homology comparison, the NR3 fragment was found homologous with DnaJ gene.

Using NR3 fragment as the probe, we screened a cDNA library of carrot somatic embryos deregulated for 12 h and acquired 18 positive phage plaques. The clones whose inserts were between 1~3 kb were selected for subcloning and sequencing. After that, we got a full-length cDNA and named it DcJ1 (GenBank accession number AF308737). Sequence analysis indicated that DcJ1 is a 1606 bp cDNA fragment with the whole 3' UTR region, a part of the 5' UTR region and a complete coding region (Fig. 2). The longest open reading frame of DcJ1 is 1257 bp, encoding a polypeptide of 418 amino acids. The predicted polypeptide has a theoretical molecular mass of 46.3 kDa and a pI of 5.18. Comparison of sequence homology showed that DcJ1 gene has 49.3% nucleotide sequence homology and 35.9% amino acid sequence homology with E. coli DnaJ. Among the yeast DnaJ family, DcJ1 has the highest homology with YDJ1/MA55, 52.5% and 45.2% for nucleotide sequence and amino acid sequence, respectively. Homology of nucleotide and amino acid sequences of DcJ1 gene with other DnaJ homologs found in higher plants is generally above 70% (Fig. 3).

2.3 Analysis of DcJ1 protein domain

Most of the DnaJ homologs isolated from plants are cytoplasmic forms\(^{7,13,14}\), others such as PCJ1 (pea chloroplast DnaJ)\(^{12}\), AtJ1\(^{11}\), AtJ6\(^{17}\) are located in chloroplast, mitochondria, and nucleus, respectively. Some common properties were found in the DnaJ homologs isolated. J-domain shared by all of the DnaJ homologs consists of approximately 75 conserved amino acid residues and comprises 4 a-helices. G/F-domain, a sequence rich in Gly and Phe residues, and CRR (Cys rich region) are rather conservative also, but they do not belong to every DnaJ homolog; and C-terminal domain is a less conserved sequence. Therefore, Cheetham et al.\(^3\) divided DnaJ homologs into 3 types: (1) those with all 3 conserved domains; (2) those with J-domain and one of G/F region or CRR; (3) those with J domain only. Furthermore, there are some characteristic sequences such as prokaryotic cell division protein FtsA, RLGS (rhodopsin-like GPCR superfamily) and ERG (erythrocruzin family) in some DnaJ homologs with J-domain only, and some of them have potential CaM-binding protein activity\(^{17}\).

Based on the homology comparison, we analyzed the protein domain of DcJ1 (Fig. 2). DcJ1 lacks the signal sequence for organelle targeting at the N-terminal, so DcJ1 may be located in the cytoplasm. DcJ1 with typical structure characteristic of DnaJ homologs comprises 3 conserved domains; the J-domain consists of 11~76aa from N-terminal, with the most conservative HPD box, this domain is responsible for the interaction of DnaJ/Hsp40 and DnaK/Hsp70\(^{22}\); the G/F region is made up of about 30aa (81~109aa), which is rich in Gly and Phe and comprises a flexible linker region that helps to convey specificity of interactions among DnaK, DnaJ, and target polypeptides; CRR (149~215aa) comprises 4 repeated CXXCGXG motifs, in which X represents amino acid residues with electric charge or polarity; this region can form a zinc finger structure, and is believed
Fig. 2. Nucleotide and deduced amino acid sequences of Dej1. The J-domain is underlined, the conserved HPD box is shaded; G/F-domain rich in Gly and Phe is in italic letters; 4 CXXC/GXG motifs in Cys rich region are marked with wavelines; putative farnesyl site CAQQ is underlined boldy.

to mediate protein-protein interactions among DnaK, DnaJ, and target polypeptides. Similar to other DnaJ homologs in eukaryotes, the GXG in the 4th repeated motif of CRR is replaced with GXK. In addition, there is a less conservative C-terminal domain, which works together with G/F region and Cys rich region to regulate the relationship between DnaJ homologs and their substrates.

The last 4 amino acid residues, CAQQ, at the C-terminal of Dej1 protein is the putative recognition site for protein farnesylation. Catalysed by farnesyltransferase (Ftase), the Cys residue of the short motif can be covalently attached by a 15-carbon farnesyl. This kind of post-translational modification is required for membrane targeting, protein-protein interactions and biological activity of key regulatory proteins of eukaryotes[19]. And it is essential to the functions of some DnaJ homologs at elevated temperatures[20]. We postulate that Dej1 proteins bind membrane by farnesylation and play their roles in cytoplasm.
2.4 Copy number of carrot DcJ1 gene

To identify the copy number of DcJ1 gene, we selected 5 kinds of restriction endonucleases to digest the genome DNA, including those that do not have any site in DcJ1 coding region, such as AciI, EcoR1, XbaI, and EcoRV and a restriction endonuclease (BglII) that has only one site in the coding region. Each group of the digested products was separated by electrophoresis on 1% agarose gels (10 μg/lane), and the products were transferred to nylon membrane. The probe that comprises intact cDNA coding region was labeled with 32P. Southern blotting analysis (Fig. 4) showed that there was 1 band on the lanes for AciI, EcoR1, XbaI and EcoRV digests. but 2 positive bands on the lane of BglII digest. This implies that DcJ1 gene may exist in the form of single copy in the carrot genome.

Recently, Miernyk[21] reported a genome approach to analyze all the J-domain proteins from the flowering plant Arabidopsis thaliana. There are a total of 89 J-domain proteins identified in the genome of A. thaliana. Based on the results of sequence comparisons and structure and function predictions, 51 distinct families were identified. The families ranged in size from 1 to 6 members. In other eukaryotes, DnaJ homologs were also identified to be multigene families and members of the families may have single or low copies[4,17].
2.5 Characteristic expression of carrot DeJ1 gene

Northern blot analysis was used to examine the expression of DeJ1 gene in different tissues of carrot. Results indicated that DeJ1 gene is expressed only in roots (Fig. 5), suggesting that carrot DeJ1 gene is a constitutive gene in roots. Because DeJ1 gene was isolated from carrot somatic embryos deregulated for 12 h, we also examined its expression during the deregulation process. Northern blotting showed that there was a certain expression level of DeJ1 gene in the regulated embryos. The expression level rose after deregulation, and hybridization signal was the strongest in the embryos deregulated for 24 h. But the expression weakened after that until the 48th hour after deregulation when the expression of DeJ1 was very low (Fig. 6). These results imply that the expression of DeJ1 gene is closely correlated to the early growth and development of embryo radicle, and it may play important roles during the development of somatic embryo radicle. Some researchers have noticed the relationship between DnaJ homologs and the development of roots. Frugis et al. [14] found that MsJ1, another DnaJ homolog in alfalfa, expresses more in roots and embryos. It seems to be related to mitosis and induction and genesis of adventitious roots. Sedbrook et al. [16] reported that a transmembrane protein ARG1, which has a J domain in Arabidopsis thaliana, interacts with cytoskeleton, and affects geotropism of roots subsequently. Our results about the characteristic expression of DeJ1 gene provide further evidence for functions of DnaJ homologs in growth and development of roots and embryos.

Fig. 5. Expression of DeJ1 gene in different tissues of carrot. 1. Leaves; 2. petioles; 3. roots.

Fig. 6. Expression of DeJ1 gene in different development stages of carrot somatic embryos. 1. Regulated embryos; 2 and 3. embryos deregulated for 24 h and 48 h.

Some studies have indicated that some DnaJ homologs can be largely induced by heat shock, but the mRNA level will drop slowly to normal as the organism adapts to the environment [7,14,15]. Based on the earlier reports [22,23], we heat-shocked the regulated carrot somatic embryos at 37 °C for periods of 30 min to 7 h. Then we extracted total RNA and checked whether the mRNA synthesis of DeJ1 gene was induced by heat shock. The result (Fig. 7) suggested that transcription of DeJ1 gene did not show evident changes during the heat shock. This implies that although DeJ1 gene belongs to the Hsp40 gene family, its function is not related to self-protection of the organism under heat stress.

In correspondence to its diversity in structure, DnaJ homologs are involved in many kinds of cellular processes. Therefore, expression of DnaJ homologs of different plant species or different DnaJ homologs in the same species is always different. Generally, they are constitutively expressed, and expression is higher in roots and cotyledons [7,14]. Under heat stress, some DnaJ homologs can be induced substantially, while some of them are not sensitive [15], e.g. DeJ1. Other stresses, such as heavy metals and salinity and so on, also can enhance expression of some DnaJ homologs [15]. Furthermore, some researchers compared the expression patterns of DnaJ homologs and DnaK homologs [7,15]. Zhu et al. [27] reported that the pattern of ANJ1 gene expression in response to heat stress was similar to that of Hsp70; however, differences in the expression of these two genes were detected during the growth cycle of suspension cultured cells. Expression of ANJ1 and Hsp70 mRNAs was also different during salinity stress. These data indicate that ANJ1 may have independent functions in addition to cooperating with Hsp70s. Corresponding evidence has been acquired in prokaryote [24], but not yet in eukaryote. We hope that through further study on DeJ1, we can gain more complete and thorough understanding on the function of DnaJ homologs.

Fig. 7. Effect of heat shock on expression of DeJ1 gene in carrot somatic embryos. 1, 26 °C, control; 2, 37 °C, 30 min; 3, 37 °C, 1 h; 4, 37 °C, 2 h; 5, 37 °C, 3 h; 6, 37 °C, 5 h; 7, 37 °C, 7 h.
The isolation of the promoter of DcJ1 gene and cis-elements that related to expression specificity in roots is of great importance for theoretical studies and practical applications.

3 Conclusion

With the improved cDNA RDA technique, we isolated a DnaJ homolog, DcJ1, from carrot somatic embryos, and performed a series of analysis on its structure and function. It is assumed that DcJ1 protein combines with the membrane, and functions in cytoplasm. The specificity of this gene lies in the fact that it is not induced by heat shock, but is a constitutive gene related to the development of roots. Isolation and analysis of DcJ1 gene not only brings a new member to the DnaJ homolog family, but also provides new evidence for the relationship between DnaJ homolog and the development of embryo radicle or growth of roots. And this study has also testified that the modified cDNA RDA method is an effective strategy to the isolation of genes related to development.

References