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Short communication

Cloning and characterisation of a carrot cDNA coding for a WD repeat protein homologous to *Drosophila* fizzy, human p55CDC and yeast CDC20 proteins

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Abstract

The present study describes the isolation of a cDNA coding for a carrot protein of 450 amino acids that contains WD repeats (DcWD1) and is homologous to *Drosophila melanogaster* fizzy protein, mammalian p55CDC and yeast Cdc20p. As for the known related proteins, sequence conservation concerned the majority of the polypeptide except the far N-terminus. Results of Southern blot analysis with genomic DNA under high stringency conditions showed the occurrence of a single gene. Northern blot analyses revealed the accumulation of DcWD1 mRNA in all tested tissues (leaves, petioles and hypocotyls, apical meristems, roots and suspension cultured cells), though at a different extent. Lack of induction of relevant transcripts in proliferating auxin-stimulated hypocotyls suggests a mode of expression not strictly related to the cell proliferation.

Cell cycle progression appears to be regulated in all eukaryotes by two major control points operating at the G_1-to-S phase (the commitment point is named START in yeast and restriction point in animal cells) and at the G_2-to-M phase boundaries [19]. In *Saccharomyces cerevisiae*, a number of cell division cycle (CDC) proteins are thought to be crucial for the orderly progression of the cell cycle. Among them, Cdc20 protein (Cdc20p) appears to be involved in the progression of mitosis. In fact, *CDC20* gene mutation is associated with abnormal microtubules and results in mitotic arrest before or during early anaphase; thus, these results suggest an involvement of Cdc20p in either disassembly or surface alteration of microtubules [18].

Mammalian p55CDC, homologous to yeast Cdc20p, has been proposed to be essential for cell division. Related transcripts are present only in proliferating cells and are not detectable in differentiated non-dividing cells. p55CDC is phosphorylated in cycling cells and belongs to a protein complex that shows kinase activity; however, p55CDC itself is not a kinase. It has been observed that the kinase activity of the complex appears to fluctuate during the cell cycle contrarily to p55CDC that remains constant [22].

Drosophila fizzy (*fzy*) gene, homologous to *S. cerevisiae CDC20*, is involved in the transition from metaphase to anaphase. The study of embryo *fzy* mutants suggests that the gene might play a role in promoting the ubiquitin-dependent proteolysis that occurs during mitosis. In contrast to wild-type yeast *CDC20*, *fzy* gene was not able to rescue the temperature-sensitive lethality of the mutant *cdc20-1*. Fizzy protein accumulates only in mitotically active cells even though *fzy* gene does not show any cell cycle-related expression during embryonic development; this sug-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U83921 (Dc WD1).

gests that a possible cell cycle regulation might occur at the protein level by post-translational modification [5].

Cdc20p, p55CDC and fizzy are part of a large group of eukaryotic proteins characterised by a variable-length N-terminal region that precedes a central domain composed of a repeating unit of about 40 amino acids (from four to eight repeats) that generally ends with Trp-Asp (WD): for this reason they are called WD repeat proteins. Within a given protein, various repeats can differ between themselves more than repeats present at the same position in differing proteins that play a similar function [14]. The WD protein family can be divided into subfamilies whose members play a role in several processes: signal transduction, regulation of cell cycle and cytoskeletal assembly, WD repeat proteins have been found to be involved also in gene regulation and development, vesicular transport and mRNA processing [14]. Such a wide functional diversity may be explained by the common role that WD repeats play in facilitating the formation of multiprotein complexes via protein-protein interactions, while the potential site for specialisation could be linked to the variable length N-terminal regions.

For plants, several cDNAs that code for WD repeat proteins have been isolated: *COP1* from *Arabidopsis thaliana*, which codes for a polypeptide with a zinc-binding motif at the N-terminus and WD motifs homologous to those of G-β proteins at the C-terminus, appears to repress photomorphogenesis in the dark and has a regulatory role [7]; ArcA from tobacco [9] and RWD from rice [10], which are homologous to Cblp from *Chlamydomonas* [17], belong to a group of receptors for activated C-kinase and appear to be involved in signal transduction; ZGB1 from maize and AGB1 from *Arabidopsis* show a high similarity to the beta subunits of G proteins [23]. The expected size of polypeptides corresponding to these ORFs ranges from 327 to 377 amino acids.

In the present study, we report for the first time the isolation and the characterisation of a carrot cDNA coding for a novel WD repeat protein (DcWD1) which is homologous to those that play a role in the progression of the cell cycle (fizzy, p55CDC, and Cdc20p). DcWD1 contains 7 WD repeats, is 450 amino acids long and shows low similarity with any other described plant WD protein. This finding contributes to the molecular insight of the plant cell cycle [4], the knowledge of which lags behind that of budding yeast and mammals.

Isolation of a cDNA homologous to yeast CDC20 *gene*

During the screening of a carrot cDNA λgt11 library with dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) cDNA as a probe (pDRTS) [13], we isolated a clone containing an insert of about 800 bp. Preliminary sequencing of the insert ends and a computer search of databases using the BLASTn program [1] revealed that the 5′ end had a 64% identity with yeast *CDC20* gene [18] over a 160 bp region, while the 3′ end was found to contain a stretch of poly(A). A similar high identity was found for the deduced amino acid sequence.

The screening of a second carrot cDNA library (λ-ZAP II cDNA library, Bernacchia and Cella, manuscript in preparation) using this clone as a probe, led to the isolation of three additional cDNA inserts, the largest of which had a size of about 2 kb and contained an ORF of 1350 bp. Analysis of the derived amino acid sequence showed the presence of seven WD repeats, the structure of which conform to the definition of WD repeat proteins given by Neer *et al.* [14] (Figure 1). The protein was named DcWD1. The comparison of DcWD1 amino acid sequence with that of *Drosophila* fizzy protein [5], mammalian p55CDC [22] and yeast Cdc20p [18], performed with the WISGEN GCG software [8], revealed a similarity respectively of 66%, 64% and 56%. In contrast, comparison of DcWD1 with known plant WD repeat proteins [7, 9, 10, 23], showed a much lower level of similarity (35–37%). Thus, these results suggest that DcWD1 is a CDC20-like protein. Results reported in Figure 1 also show that complete sequence divergence occurs at the far N-terminus while the highest similarity is located at the C-terminus which contains WD-40 repeat motifs [14]; among the latter, the second and third repeats show a relatively high degree of degeneration.

Gene copy number

In order to see if DcWD1 was the product of a single gene or of a gene family, carrot genomic DNA was digested with one or a combination of two restriction enzymes, and analysed by Southern hybridisation using the 800 bp cDNA as a probe. Results of this experiment showed the presence of a single band, thus suggesting the presence of a single gene (Figure 2).

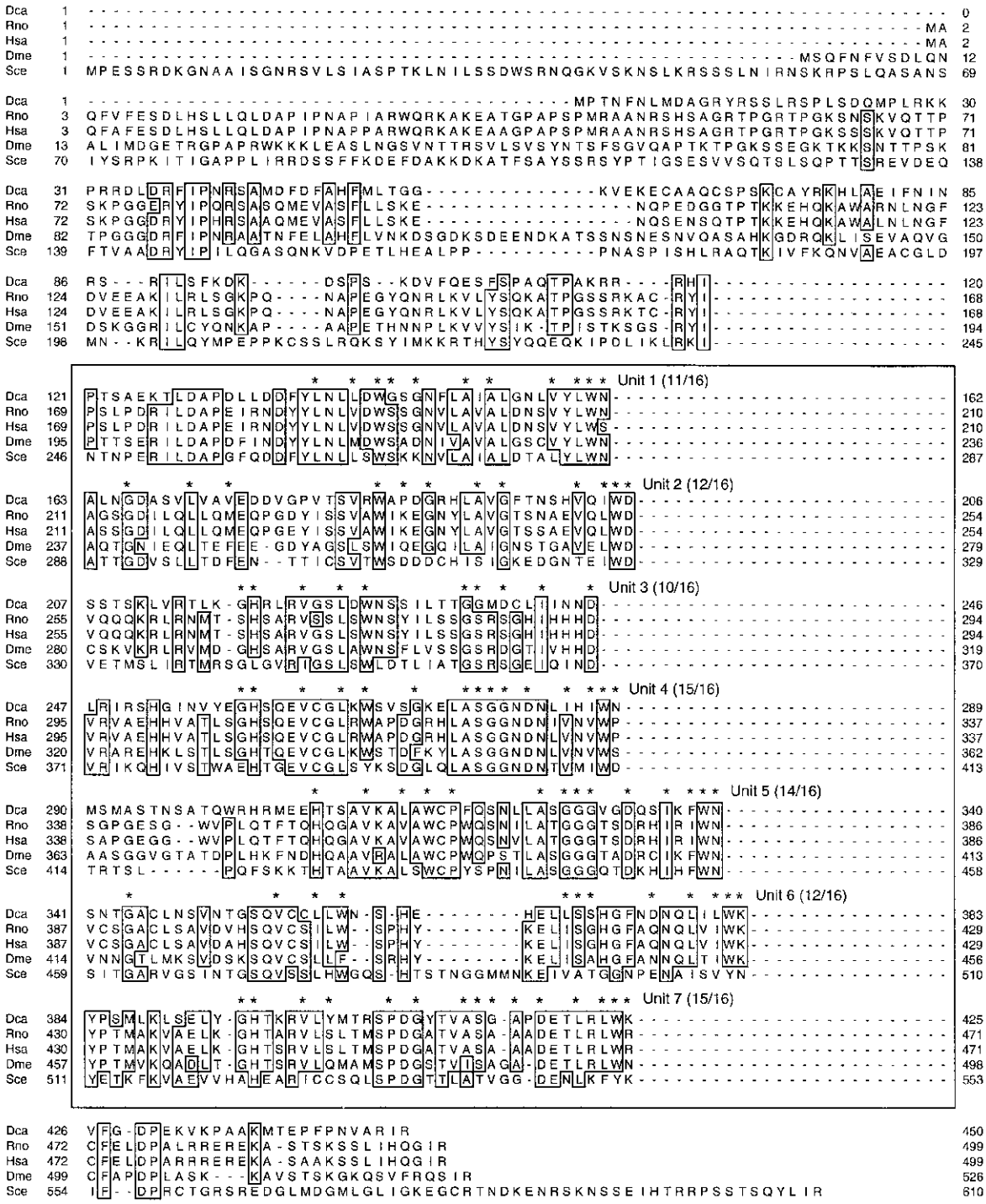

Figure 1. Comparison of the deduced amino acid sequence of DcWD1 with the best matched sequences of proteins coded by rat and human genes for p55CDC (Rno and Hsa), *Drosophila fzy* gene (Dme) and budding yeast *CDC20* (Sce). The region of the protein containing the conserved WD repeats is boxed and single units numbered. Asterisks indicate amino acids conserved in the carrot protein in accordance with the consensus defined by Neer *et al.* [14]. Values in brackets indicate the number of conserved residues per unit. Two carrot cDNA libraries (λgt11 [13] and λ-ZAP II; Bernacchia and Cella, manuscript in preparation) were screened with [^{32}P]-labelled probes in accordance with standard protocols [16]. The cDNA inserts were obtained from positive λgt11 cDNA clones by PCR with the forward and reverse vector primers, filled in with PolIk and bluntly ligated to pGEM-4Z (Promega). The inserts from λ-ZAP cDNA clones were directly subcloned into pBluescript phagemid (Stratagene) by *in vivo* excision in accordance with the manufacturer's instructions. DNA sequencing was performed by the dideoxynucleotide technique using T7 sequencing kit (Pharmacia) with [^{35}S-dATP]. When needed, 17-mer oligonucleotides were synthesised and used as sequencing primers. Sequence data were analysed with MacDNASIS v3 (Hitachi), BLASTn algorithm [1] and CLUSTAL W software [20].

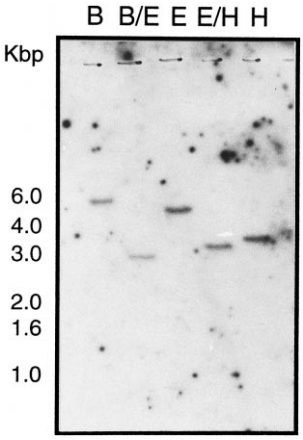

Figure 2. Analysis of the gene copy number. Carrot genomic DNA was extracted from suspension cells as described [6] and digested with *Bam*HI (B), *Bam*HI/*Eco*RI (B/E), *Eco*RI (E), *Eco*RI/*Hin*dIII (E/H) and *Hin*dIII (H). Aliquots of 5 μg of carrot genomic DNA were digested as indicated and blotted onto Hybond N^+ (Amersham). Probes were labelled with DIG DNA labelling kit (non-radioactive labelling system, Boehringer) in accordance with the manufacturer's instructions. Hybridisation was carried out under standard conditions. The filter was washed at 65 °C twice for 5 min with 2× SSC/0.1% SDS and twice for 15 min with 0.1× SSC/0.1% SDS. The signal was detected using an anti-digoxigenin antibody/alkaline phosphatase conjugate in accordance with the manufacturer's instructions (Boehringer).

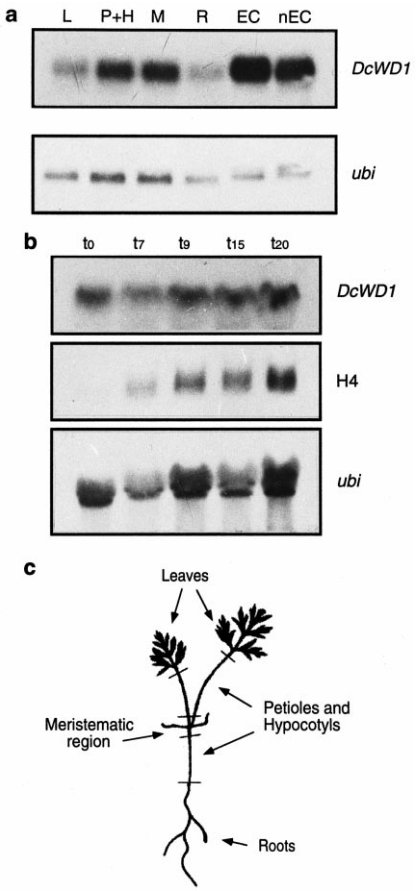

Figure 3. Accumulation of DcWD1 mRNA in carrot tissues and suspension-cultured cells. a. Poly $(A)^+$ mRNA was extracted by oligo dT chromatography [3] from 40-day-old carrot plant tissues (L, leaves; P+H, petioles and hypocotyls; M, meristematic region; R, roots; as illustrated in (c) and from suspension cell cultures, both embryogenic (EC) and non-embryogenic (nEC)). Aliquots of 2 μg were separated by denaturing agarose gel electrophoresis [16], blotted onto nylon membranes and hybridised under standard conditions with [^{32}P]-labelled cDNA coding for DcWD1 as a probe. b. Total RNA was extracted from both untreated and 2,4-D stimulated hypocotyl segments in accordance with Verwoerd *et al.* [21]. Stimulation of cell proliferation was induced as described by LoSchiavo *et al.* [12] by growing hypocotyl segments in the presence of 0.5 mg l^{-1} 2,4-D for differing times (7 days = t_7, 9 days = t_9, 15 days = t_{15}, 20 days = t_{20}). Aliquots of 30 μg of RNA were electrophoresed and hybridised with *DcWD1* and *A. thaliana* H4 (EST 122P14T7A, GenBank accession number T44161) probes as described in a. Both filters were hybridised with a parsley ubiquitin cDNA (*ubi*) [11]. c. Schematic representation of a 40-day-old carrot plantlet.

Expression of the DcWD1

Northern blot analysis was performed with poly$(A)^+$ RNA extracted from different tissues of 40-day-old plants and actively dividing suspension-cultured cells: these plant materials are characterised by a widely differing mitotic index (namely, negligible in hypocotyls and high in meristems and suspension cells). Results of this experiment, which are reported in Figure 3a, indicate that DcWD1 mRNA (about 2000 nt) accumulates preferentially in proliferating suspension cells and apical meristems (Figure 3a, EC, nEC and M); however, it is also present, though at a lower level, in differentiating tissues that are characterized by a much lower level of cell proliferation (Figure 3a, P+H, L and R). To better analyse the relationship between DcWD1 gene expression and cell division, we followed the accumulation of the relevant transcript in auxin-stimulated hypocotyl fragments: these are characterised by a massive resumption of cell proliferation induced by 2,4-dichlorophenoxy acetic acid (2,4-D) at the level of pro-cambial cells that results in callus formation at the edge of explants [12]. Results of this experiment, which are reported in Figure 3b, show that the level of DcWD1 mRNA did not increase upon exposure to auxin and its amount was similar to that of untreated hypocotyls. Contrastingly, under the same conditions, histone H4 (H4) mRNA accumulation was significantly stimulated (Figure 3b). A similar beha-

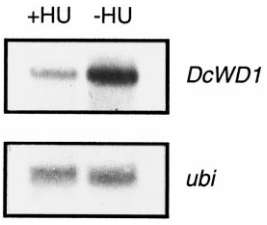

Figure 4. Down-regulation of DcWD1 mRNA in carrot suspension cell treated with hydroxyurea. Actively growing cells were grown for 72 h either in the absence (−HU) or in the presence of 100 mM hydroxyurea (+HU) and then collected by filtration. Poly(A)$^+$ mRNA was extracted, electrophoresed and hybridised as described in Figure 3.

viour was observed for carrot DNA topoisomerase I gene whose expression in hypocotyls is stimulated by 2,4-D and shows a clear correlation with cell proliferation [2].

Northern analysis with poly(A)$^+$ RNA extracted from suspension cells treated for 48 h with hydroxyurea showed a significant reduction of DcWD1 mRNA (Figure 4). This behaviour is similar to that of the gene coding for tobacco histone H4 whose transcripts almost disappear upon treatment with hydroxyurea [15].

Conclusions

Results presented in this study describe the isolation of a cDNA that codes for a novel WD repeat protein (DcWD1) homologous to those proteins that play a role in the progression of the cell cycle (fizzy, p55CDC, and Cdc20p). DcWD1, which contains seven WD repeats, has a size of 450 aa and is the smallest protein of this class described so far.

Northern blot analysis revealed that the gene for DcWD1 is expressed in both proliferating and differentiating tissues. Particularly, lack of induction of *DcWD1* gene in auxin-stimulated hypocotyls would suggest a mode of expression not directly related to cell division. The expression pattern of *DcWD1* gene contrasts with that of genes coding for Cdc20p, p55CDC and fzy protein, whose transcripts are detectable only in mitotically active cells. It also differs from that of other carrot genes such as *top1* and histone H4 that show up-regulation upon induction of cell proliferation mediated by phytohormones.

Interestingly, *DcWD1* gene is down-regulated upon treatment with hydroxyurea similarly to tobacco histone H4 gene whose expression was proposed to be dependent on G1/S transition but not on DNA replication on the basis of experiments using protoplasts treated with aphidicolin or hydroxyurea [15].

A possible explanation of this puzzling behaviour is that, at least in young plantlets used in this study, *DcWD1* gene might be regulated post-transcriptionally, or post-translationally as it has been proposed for fzy protein [5].

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