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Isolation and characterization of homeobox-containing genes of carrot

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Abstract

Homeodomains (HDs) are DNA-binding domains that have been well characterized in animals, and HD proteins are thought to be regulators of transcription. To investigate the regulation of gene expression during somatic embryogenesis in carrot, an attempt was made to isolate cDNA clones that encode HD proteins. A cDNA library from carrot somatic embryos was screened with a degenerate oligonucleotide probe that corresponded to a conserved amino acid sequence of HDs, and one cDNA clone (CHB1) encoding an HD protein was isolated. The amino acid sequence deduced from the nucleotide sequence of this clone contained a putative leucine zipper motif adjacent to the anticipated HD. The homeodomain/leucine zipper (HD-Zip) sequence of this cDNA was used for further screening, and five additional independent clones (CHB2 through CHB6) were isolated. Although the HD-Zip sequences encoded by these clones were similar to each other, the sequences beyond the HD-Zip regions varied greatly. Transcripts corresponding to CHB1 through CHB6 were expressed at different times during somatic embryogenesis. In particular, transcripts corresponding to CHB2 were expressed in close association with the early development of embryos.

Introduction

The homeobox (HB), a small DNA segment of 183 bp that encodes a polypeptide known as a homeodomain (HD), was first found as a sequence that was characteristic of homeotic genes of *Drosophila*. These genes are master control genes that play a key role in the specification of the body plan [9]. Numerous results accumulated over the past few years have shown that HD proteins function as transcriptional regulators and that the HD is responsible for sequence-specific binding to DNA via a helix-turn-helix motif [10, 18, 27]. HB sequences have been found in a variety of species, and they can be classified into at least 30 distinguishable groups from comparisons

The nucleotide sequence data reported will appear in the GSDB, DDBJ, EMBL and DNCBI Nucleotide Sequence Databases under the accession numbers D26573 (CHB1), D26574 (CHB2), D26575 (CHB3), D26576 (CHB4), D26577 (CHB5) and D26578 (CHB6).

of their amino acid sequences [14]. Homeobox genes in vertebrates are homologous to homeotic genes in *Drosophila*, and the encoded proteins function in pattern formation associated with the development of tissues and/or organs. It now appears that the mechanisms responsible for the genetic control of development are much more universal than has been anticipated [10].

In higher plants, several HB genes have been cloned since their first identification in maize [31] and *Arabidopsis* [23]. The *Knotted-1* (*Kn1*) gene in maize, a gene in which dominant mutations alter patterns of leaf development, was cloned by transposon tagging [11] and found to encode an HD protein [31]. The pattern of expression of the transcript and the product of translation of *Kn1* has been characterized [30]. The function of the Kn1 protein was analyzed by an examination of the phenotype of transgenic tobacco plants in which *Kn1* was overexpressed [29]. It appears that *Kn1* plays a role in regulating the switch from indeterminate to determinate cell fate [29, 30]. A gene homologous to *Kn1* has been isolated from rice [13, 20]. Furthermore, the screening of cDNA expression libraries with well characterized *cis*-acting elements as probes allowed isolation of cDNA clones that encoded HD proteins from maize and *Arabidopsis* [1, 26]. In the case of *Arabidopsis*, several cDNAs for proteins that contained an HD were cloned using degenerate oligonucleotides designed on the basis of the conserved amino acid sequence of the HD [21, 23, 24]. In addition to the HD, all of the cDNAs encoded a leucine zipper motif, a characteristic motif of transcription factors, adjacent to the carboxy-terminal region of the HD. Recent studies of the HB genes from plants have suggested that their products have DNA-binding activities and function in the regulation of plant growth [4, 25, 28].

Somatic embryogenesis in carrot is an ideal system for investigations of the entire process of development in plants [for review, 32]. Using this system, many efforts have been made to isolate genes that regulate embryogenesis. However, the products of most of the genes that have been isolated are not involved directly in the regulation of embryogenesis but, rather, they are involved in metabolism and cellular structure. The HD sequence is universally conserved in both the animal and the plant kingdom, and it is anticipated that the importance of HD proteins will be recognized in the development of both animals and plants.

In this report, we describe the isolation of genes for HD-containing proteins and the characterization of their expression during somatic embryogenesis in carrot. A cDNA clone containing an HB was isolated from a somatic embryo cDNA library by use of a degenerate oligonucleotide probe. The deduced amino acid sequence encoded by this clone contained a leucine zipper motif adjacent to the carboxy-terminal region of the HD. The sequence encoding the homeodomain-leucine zipper (HD-Zip) was used for further screening, and five additional independent clones were isolated. Northern blot analysis revealed that the corresponding mRNAs were expressed at different times during somatic embryogenesis.

Materials and methods

Plant materials and culture methods

The cell culture was derived from the hypocotyl of a seedling of the domestic carrot (*Daucus carota* L. cv. Kurodagosun). The cells were subcultured at 7-day intervals in a modified version of Lin and Staba's medium [5], which contained 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 μM zeatin. The cells in suspension culture were grown at 27 °C in 120 ml of medium in 500 ml conical flasks on a reciprocal shaker (90 strokes/min and 5 cm amplitude) in darkness.

Embryogenesis was induced by the method of Fujimura and Komamine [6] with slight modifications. In brief, clusters of embryogenic cells of 32 to 50 μM in diameter, referred to as State-1 cell clusters, were fractionated by sieving and density gradient centrifugation, and then they were cultured in a modified version of Lin and

Staba's medium that contained 0.5 μM 2,4-D for three days. Then the cell clusters were washed to remove 2,4-D with the basal medium without growth regulators. The washed clusters were cultured in the same basal medium for induction of embryogenesis, or in the basal medium that supplemented with 0.5 μM 2,4-D to generate undifferentiated cell clusters. The cells suspended in 200 ml of one or the other medium were incubated in 500 ml conical flasks on a reciprocal shaker (90 strokes/min, 5 cm amplitude) at 27 °C in darkness. 2,4-D was added as a 200 μl aliquot of a 0.5 mM solution of 2,4-D to 200 ml of the culture medium 6 days or 8 days after the start of induction of embryogenesis. The final concentration of 2,4-D was 0.5 μM. For controls, 200 μl of H_2O were added instead of the solution of the 2,4-D.

Preparation of RNA

Total RNA was extracted from the cells that has been cultured in medium with or without 2,4-D and from carrot seedlings by the method described by Ozeki *et al.* [22]. Poly(A)-rich RNA was isolated from total RNA by two cycles of affinity chromatography on oligo(dT)-cellulose [19].

Cloning of cDNA

For construction of a cDNA library for somatic embryos, double-stranded cDNA was synthesized from poly(A)-rich RNA isolated from somatic embryos, at the late globular and early heart-shaped stages, harvested 6 days after the start of induction of embryogenesis with a cDNA synthesis kit (Pharmacia LKB Biotechnology, Tokyo, Japan). The double-stranded cDNA was cloned into λgt11. About 10^6 recombinants were packaged *in vitro*. The ratio of non-recombinants to recombinants, as determined by color selection [19], was about 5%. A λgt11 cDNA library for carrot hypocotyls was kindly provided by Dr M. Matsumoto (Shionogi, Shiga, Japan). Screening of the somatic embryo cDNA library with oligonucleotide HB-1 (AA(A/G)ATXTGGTT(T/C)-CA(A/G)AA(C/T)(A/C)GX(A/C)G) [3] was performed as described by Burglin *et al.* [3]. Clones CHB2 and CHB3 and clones CHB4, CHB5 and CHB6 were obtained by rescreening of the somatic embryo and hypocotyl cDNA libraries respectively, with a *Fok* I fragment that contained a sequence that corresponded to the HD-Zip region of CHB1. For this screening, an ECL direct nucleic acid labeling and detection system (Amersham Life Science, Tokyo, Japan) was used. Hybridization and detection were performed in accordance with the supplier's protocol. The phage DNA was isolated by the method of Maniatis *et al.* [19], and the cDNA inserts were subcloned into the *Eco* RI or *Not* I site of the plasmid vector BlueScript KS (Stratagene, Heidelberg, Germany).

RNA gel-blot analysis

Northern blotting was performed as described by Berk *et al.* [2] with slight modifications. Total RNA was fractionated on an agarose gel and then bands of RNA were transferred to a nylon membrane (Biodyne A; Nihon Pall, Tokyo, Japan) by capillary blotting. The cDNA inserts were labeled with ^{32}P by use of a Megaprime DNA labeling system (Amersham Life Science). Hybridization and washing of filters were performed in accordance with the protocol from the supplier of the filters.

Sequencing of DNA

The cDNA inserts that had been subcloned in the plasmid vector were sequenced by the dideoxy chain-termination technique using Sequenase Ver. 2.0 (United States Biochemical, Cleveland, OH) or the Taq Dye Primer Cycle Sequencing Kit and a model 373A DNA sequencer (Applied Biosystems Japan, Tokyo, Japan) in accordance with the respective suppliers' protocols. Deletion clones were prepared with a Double-Stranded

Nested Deletion Kit (Pharmacia) as indicated by the supplier.

Analysis of sequences was performed with the SDC-Genetyx program (Software Development Co., Tokyo, Japan).

Results

Isolation of cDNA clones that contained a homeobox sequence

To isolate cDNA clones that contained a homeobox sequence, a somatic embryo cDNA library was screened by the method developed by Burglin *et al.* [3]. They designed five degenerate oligonucleotide probes (23 bases long) that corresponded to the highly conserved region of the helix-3 region of the HD, and then they screened genomic libraries of *Caenorhabditis elegans* under hybridization conditions that yielded strong hybridization to DNA fragments with two or fewer mismatches. We screened a cDNA library derived from somatic embryos under the same conditions with one of the probes, HB-1, which was designed to detect a large set of HB-containing genes [3]. About 120000 plaques were screened, and six independent clones were obtained. Only one of the six clones, designated CHB1, was found to encode an HD protein, and it also encoded a leucine zipper motif. Further screening of somatic embryo and hypocotyl cDNA libraries was carried out to obtain clones other than CHB1 using a DNA fragment that corresponded to the HD-Zip region of the CHB1 protein as the probe, since little similarity is to be expected among the primary sequences outside the HD in HD proteins [27]. As a result, five additional independent clones (CHB2 and CHB3 from the embryo library, CHB4, CHB5 and CHB6 from the hypocotyl library) were obtained.

Sequence analysis of the CHB series of cDNA clones

The nucleotide sequences of the six CHB clones were determined. CHB1 cDNA of 1599 bp, excluding the poly(A) tail, included one long open reading frame of 621 bp that encoded a protein of 206 amino acids. There was a difference of two nucleotides between the CHB1 sequence and the oligonucleotide used as the probe for screening. In the HD sequences characterized to date, there are four invariant amino acid residues and several highly conserved amino acid residues ([27], shown in Fig. 1 as HB-CONS). The amino acid sequence deduced from CHB1 contained all the invariant amino acid residues and 11 of 17 highly conserved amino acid residues in the predicted HD region (Fig. 1). In addition, a leucine zipper motif, characterized by heptad repeats of leucine residues and thought to function during formation of dimers with other transcription factors [17], was found adjacent to the HD (Fig. 1). The lengths of the other cDNAs, excluding the poly(A) tail of each, were 1276 bp (CHB2), 1430 bp (CHB3), 1092 bp (CHB4) and 1126 bp (CHB5). Each cDNA also included a long open reading frame of 906 bp (CHB2), 840 bp (CHB3), 927 bp (CHB4) and 789 bp (CHB5), and the respective deduced amino acid sequences contained HD-Zip regions. The 1053 bp insert of CHB6 did not have a poly(A) tail and lacked the *Eco* RI linker sequence at one end. However, an HD-Zip sequence was found, in frame, starting with an ATG at position 602 from the end of the insert at which the *Eco* RI linker had been attached. The downstream sequence containing a poly(A) tail was probably lost during construction of the cDNA library.

Similarities among various HD-Zip clones

The HD-Zip sequences encoded by the CHB clones were compared with those encoded by seven other clones namely, Athb-1, Athb-2 [23], Athb-3 [21], Athb-4 [4] and HAT4, HAT5, and HAT22 [24] from *Arabidopsis*. Alignment of amino acid sequences is shown in Fig. 1. Generally, the amino acid sequences of the HDs were found to be very similar to each other, whereas less similarity was exhibited among the leucine zipper motifs. The HD-Zip sequences encoded

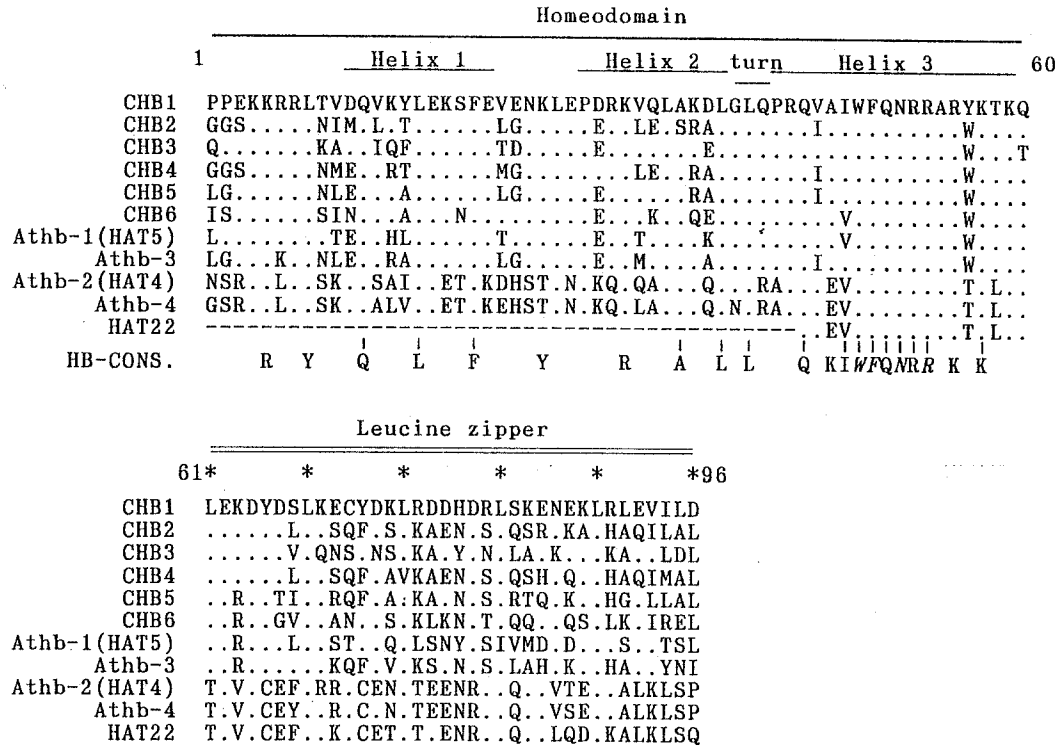

Fig. 1. Comparison of the amino acid sequences of HD-Zip regions encoded by CHB1 to CHB6 with HD proteins from *Arabidopsis*. Dots indicated identical aminoacids in CHB1 and other sequences. HB-CONS. shows the highly conserved and invariant (italics) amino acids in the homeodomains characterized to date [27]. The positions of leucine residues in the leucine zipper motif are indicated by asterisks.

by all the CHB clones were more similar to those of Athb1 (HAT5) and Athb3 (more than 60% amino acids are identical) than to those of Athb2 (HAT4), Athb4 and HAT22 (less than 46% similarities). There was no significant similarity among sequences outside the HD-Zip regions among CHB clones or between CHB clones and other HD-containing clones (data not shown) with the exception of CHB2 and CHB4. Approximately 73% of the amino acids encoded by CHB2 and CHB4 were identical but several gaps had to be introduced to maximize alignment (data not shown).

Patterns of expression of the CHB transcripts

Northern blot analysis was performed to characterize the patterns of expression of the six CHB mRNAs during somatic embryogenesis of carrots and in organs of carrot seedlings (Fig. 2). Full-length cDNAs of six CHBs used as probes did not cross-hybridize each other. The hybridization of probes indicated that the approximate sizes of the mRNAs were 1.7 kb for CHB1, 1.5 kb for CHB2, 1.6 kb for CHB3, 1.3 kb for CHB4, 1.3 kb for CHB5 and 2.3 kb for CHB6. Transcripts corresponding to each clone exhibited reproducibly a different pattern of expression. The CHB1 transcript was expressed at a constant level throughout embryogenesis and in undifferentiated cell clusters, while lower levels of expression were detected in each organ of seedlings. The level of the CHB2 transcript increased after the globular stage with a peak at the heart-shaped and early torpedo-shaped stages. Only trace amounts of the CHB2 transcript were detected in undifferentiated cell clusters. Thus, CHB2 transcripts accu-

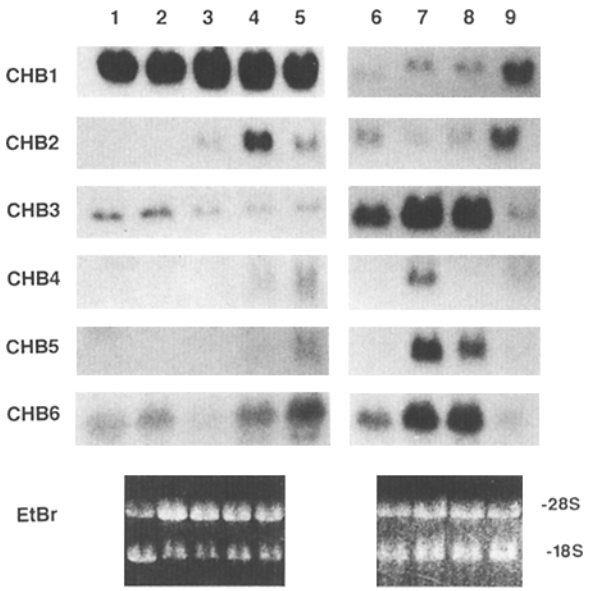

Fig. 2. RNA blot analysis of the expression of mRNAs that correspond to CHB clones. Each lane was loaded with 8 μg (lanes 1–5) or 10 μg (lanes 6–9) of total RNA. Lane 1, undifferentiated cell clusters cultured for 6 days in 2,4-D-containing medium; lanes 2–5, somatic embryos harvested 3 days (preglobular embryos, lane 2), 6 days (globular and early heart-shaped embryos, lane 3), 9 days (heart-shaped and early torpedo-shaped embryos, lane 4) and 14 days (torpedo-shaped embryos; lane 5) after the induction of embryogenesis; lane 6–8, extracts of the cotyledon (lane 6), hypocotyl (lane 7) and root (lane 8) of a carrot seedling; lane 9, the same extract as in lane 4. Note that exposure time was 3 days (CHB1–CHB3) and 8 days (CHB4–CHB6) for the hybridization in lanes 1–5, and 1 day (CHB1), 3 days (CHB2, CHB3 and CHB6) and 6 days (CHB4 and CHB5) for the hybridization in lanes 6–9. EtBr, the photograph of ethidium bromide-stained gel for CHB2 hybridization, indicating the equal loading of RNA. Similar photographs showing equal loading of RNA was also obtained for the gels used for the hybridization with the other CHB clones.

mulated specifically at the heart-shaped and early torpedo-shaped stages during embryogenesis. In seedlings, the CHB2 transcript was present at the highest level in cotyledons. The pattern of expression of the CHB3 transcript was a mirror image of that of the CHB1 transcript, that is, the CHB3 transcript accumulated at higher levels in each organ of seedlings than in cultured cells or embryos. The CHB4 and CHB5 transcripts were not detectable in somatic embryos at the early stages or in undifferentiated cell clusters, but their levels increased as the development of embryos advanced. In seedlings, the CHB4 transcript was found almost exclusively in hypocotyls, while the CHB5 transcript was detected not only in hypocotyls but also in roots. The CHB6 transcript was detectable in undifferentiated cell clusters and preglobular embryos, decreased transiently at the globular and early heart-shaped stage, and increased again thereafter. In seedlings, the CHB6 transcript accumulated in all organs.

Effects of an auxin on the expression of the CHB transcripts

Auxins are known to be potent inhibitors of embryogenesis [5, 7, 8, 12]. Fujimura and Komamine [8] reported that embryogenesis was inhibited completely by 2,4-D when it was added at the very early stage of embryogenesis, and that 2,4-D was less inhibitory to embryogenesis when it was added at the later stages of culture. Under our culture conditions, the addition of 2,4-D 6 days after the start of the induction of embryogenesis suppressed the progress of embryogenesis and caused the unorganized growth of cells in embryos. Two days after the addition of 2,4-D to a culture of globular and heart-shaped embryos, the embryogenesis ceased and swollen embryos appeared. By contrast, torpedo-shaped embryos developed at the same time in control cultures to which H_2O had been added instead of 2,4-D. To analyze the details of the disruption of embryogenesis by 2,4-D at the molecular level, the effects of treatment with 2,4-D on the levels of transcripts of CHB1, CHB2 and CHB3 were examined (Fig. 3A). The addition of 2,4-D to embryos that had been cultured for 6 days slightly increased the level of the CHB1 transcript. In contrast, the CHB2 transcript became undetectable one day after the addition of 2,4-D. Detailed analysis indicated that the level of the CHB2 transcript decreased rapidly within 1 hour after the addition of 2,4-D (Fig. 3B). 2,4-D had no effect on the expression of the CHB3 transcript.

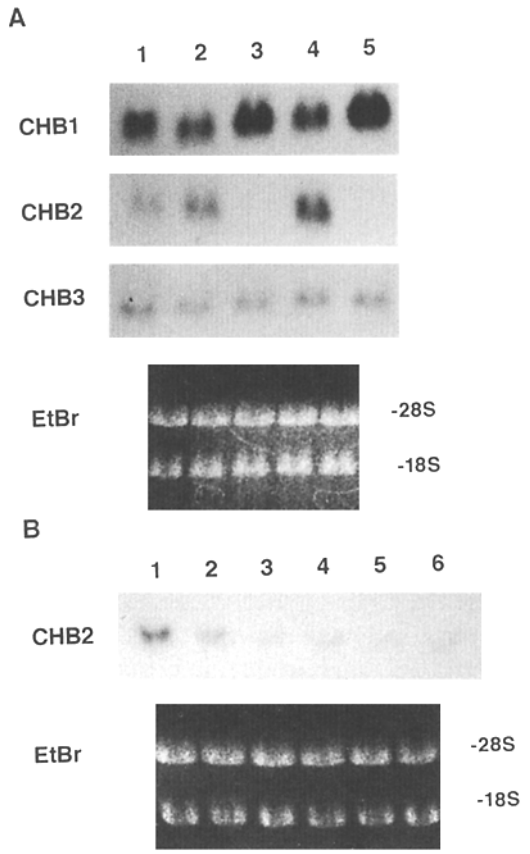

Fig. 3. RNA blot analysis of the effect of 2,4-D on the expression of the CHB1, CHB2 and CHB3 transcripts. Each lane was loaded with 10 μg of total RNA. A. Lane 1, globular and early heart-shaped embryos harvested 6 days after the induction of embryogenesis; lane 2 and 3, embryos harvested 1 day after addition of H_2O (lane 2) or 2,4-D (lane 3) on the sixth day after the start of induction of embryogenesis; lanes 4 and 5, embryos harvested 2 days after addition of H_2O (lane 4) or 2,4-D (lane 5) on the sixth day after the start of induction of embryogenesis. EtBr, the photograph of ethidium bromide-stained gel for CHB3 hybridization, indicating the equal loading of RNA. Similar photographs showing equal loading of RNA was also obtained for the gels used for the hybridization with the other CHB clones. B. 2,4-D was added 8 days after the start of induction of embryogenesis (lane 1) and embryos were harvested 1 (lane 2), 2 (lane 3), 4 (lane 4), 6 (lane 5) and 8 (lane 6) hours after this addition of 2,4-D. EtBr, the photograph of ethidium bromide-stained gel indicating the equal loading of RNA.

Discussion

Sequences of proteins encoded by the CHB cDNAs

We succeeded for the first time in isolating six cDNAs (CHB1–CHB6) that encoded HD-Zip proteins from carrot using a degenerate oligonucleotide probe that corresponded to the highly conserved sequence of the HD and subsequent cross-hybridization. Comparison of the deduced amino acid sequences of the CHB cDNAs with those of cDNAs for HD-Zip proteins from *Arabidopsis* indicated that the CHB cDNAs encoded novel HD-Zip proteins. Alignment of putative amino acid sequences (Fig. 1) showed that the WFQNRRAR motif is common to the HD sequences of all the CHB proteins and all the HD-Zip proteins of *Arabidopsis* characterized to date [21, 23, 24, 4]. Because this motif is not conserved in the plant HD proteins that do not contain the leucine zipper sequence [1, 26, 31], the WFQNRRAR motif may be characteristic of the HD-Zip proteins of plants. From genomic Southern blot analysis, Ruberti *et al.* [23] estimated that the *Arabidopsis* genome could code at least 15 HD proteins that are characterized by the WFQNRR motif in the recognition helix. Therefore, carrot may also have many other HD-Zip proteins, including homologues of the known HD-Zip proteins of *Arabidopsis*. On the basis of sequence similarity, Carabelli *et al.* [4] grouped the HD-Zip proteins into two different families: HD-ZIP I, which included Athb-1, HAT5 and Athb-3; and HD-ZIP II, which included Athb-2, HAT4, Athb-4 and HAT22. The results of our comparison of encoded amino acid sequences (Fig. 1) allows to assign all the CHB-encoded proteins to the HD-Zip I family.

Expression and function of CHB-encoded proteins

Northern blot analysis indicated that the patterns of expression of CHB transcripts were very different from one another during somatic embryogenesis in carrot and in each organ of seedlings. The transcript of CHB1 was expressed both in

somatic embryos and in undifferentiated cell clusters. The pattern of expression of the CHB1 transcript was similar to that of transcripts of a gene for elongation factor 1α (EF-1α), with the exception that lower levels of transcripts for EF-1α were found in undifferentiated cell clusters and in torpedo-shaped embryos [15]. Hybridization analysis *in situ* has indicated that the gene for EF-1α is expressed preferentially in meristematic regions, and a decrease in the level of the transcript of this gene in torpedo-shaped embryos was interpreted to be the result of the presence of only limited meristematic regions in the embryos [16]. Since the level of the CHB1 transcript was low in seedlings but high even in torpedo-shaped embryos and undifferentiated cell clusters, the CHB1 transcript may be expressed in undifferentiated and incompletely differentiated cells, and the translated protein may function in maintaining an indeterminate cell fate, as dose the Kn1 protein in maize [29, 30].

The CHB2 transcript was very specifically expressed at the heart-shaped to the early torpedo-shaped stage during somatic embryogenesis. Upon the addition of 2,4-D at the globular to heart-shaped stage, the expression of the CHB2 transcript was suppressed within 1 hour and, subsequently, embryonic development was inhibited and embryos became disorganized. This is the first report, to our knowledge, that the transcript of the gene for a possible transcription-regulatory factor is expressed in close association with a specific stages of embryonic development in plants. The heart-shaped stage is one of the most important stages of embryonic development because, at this stage, various specific types of tissue start to be generated. Therefore, the specific expression of the CHB2 transcript in heart-shaped and early torpedo-shaped embryos suggests that the CHB2-encoded protein might act as a regulator of transcription to control the expression of certain genes whose products function in the organization or differentiation of tissues in embryos. Although the CHB2 transcript was also accumulated in seedlings, those levels were lower than that in heart-shaped and early torpedo-shaped embryos. The low-level expression may be due to the continuous organization or differentiation of tissues in seedlings.

The CHB3 transcript accumulated at higher levels in every organ of seedlings than in undifferentiated cell clusters and in developing embryos. This result suggests that the CHB3-encoded product functions preferentially in mature tissues of seedlings.

The levels of transcripts of CHB4, CHB5 and CHB6 increased at later stages of embryonic development, and the transcripts were accumulated in some organs of seedlings. The CHB4 transcript was detected specifically in hypocotyls of seedlings, as well as in embryos at later stages, suggesting that the CHB4 protein is expressed specifically in the developing hypocotyl and regulates the expression of hypocotyl-specific genes. Because the CHB5 transcript was expressed not only in hypocotyls but also in roots of seedlings, the CHB5-encoded product may act in the regulation of the formation of tissues common to both organs. The CHB6 transcript was expressed in all cultured cells and organs examined with the exception of globular and early heart-shaped embryos. This pattern was unique, but the involvement of the CHB6-encoded product in embryogenesis remains to be analyzed.

Recently, the DNA-binding properties of the HD-Zip domain of an HD-Zip protein of *Arabidopsis* was analyzed *in vitro*. The HD-Zip domain of the protein product of Athb-1 bound to DNA as a homodimer [28]. Considering the high degree of similarity between HD-Zip sequences of CHB-encoded proteins and the protein encoded by Athb-1, we suggest that CHB-encoded proteins might function as transcriptional regulators by binding to DNA as dimers. Although the HD-Zip domain of the Athb-1 protein did not interact with the HD-Zip domain of the Athb-Z protein [28], the CHB-encoded proteins may bind to DNA not only as homodimers but also as heterodimers, because the similarities in terms of HD-Zip domains among CHB-encoded proteins are higher than the similarity between those domains in the Athb-1 and Athb-2 proteins. Formation of heterodimers with different combinations of CHB-encoded proteins might be

responsible for the complex regulation of gene expression during embryogenesis or the development of seedlings.

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