

Isolation and characterization of cDNA encoding P-19.5 protein accumulated preferentially at early stage of carrot somatic embryogenesis

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Abstract

We found P-19.5 protein that preferentially accumulated in cell clusters and globular embryos at the early stage of carrot somatic embryogenesis. P-19.5 protein was isolated from 9-day-old cell clusters with SDS-PAGE, and then determined the amino acid sequences of a N-terminal peptide and some internal peptides. Using each primer for these peptides, the full-length sequence of cDNA encoding P-19.5 protein was determined by analyzing 3'-RACE and 5'-RACE products. We regard P-19.5 protein as an allergen-like protein because the P-19.5 protein sequence predicted from cDNA was homologous to celery major allergen (75%), carrot major allergen (70–72%), *Pimpinella brachycarpa* pathogenesis-related protein (71%), and parsley pathogenesis-related protein (63%). We found concomitantly two separate cDNAs (*P-16* and *P-19.5 H* cDNAs) during analyzing *P-19.5* cDNA: *P-16* cDNA was cloned and isolated from P-16 protein that was recognized against P-19.5 protein antiserum, and *P-19.5 H* cDNA that had homology (80.5% amino acid identity) with *P-19.5* cDNA was detected during analyzing *P-19.5* cDNA. Northern blotting revealed that *P-19.5* and *P-19.5 H* transcripts were highly expressed at early stages of cell cluster and globular embryos, whereas *P-16* transcript was invariably expressed at all stages from cell clusters to plantlets.

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1. Introduction

In a previous paper [1], we reported on characterization of no. 43 cDNA clone whose transcripts accumulated preferentially in cell clusters, stable intermediate multicellular structures between somatic cells and embryos. This no. 43 cDNA clone shared a high degree of homology with thaumatin-like protein. Thaumatin-like protein was referred to as PR-like proteins that accumulate in response to stress, such as wounding, infection by a virus or fungus and under osmotic stress [2–4]. In somatic embryogenesis of *Cichorium*, three PR proteins (β -1,3-glucanase, chitinase, and osmotin-like protein) were found to show a

higher accumulation in embryogenic culture medium than in non-embryogenic culture medium [5]. In addition, Lin et al. [13] isolated and characterized two genes encoding PR proteins among many genes that were expressed during carrot embryogenesis. Thus, PR proteins may have an important and essential involvement in the embryo-forming mechanism, though their biochemical function in somatic embryogenesis is not yet known.

In the present paper, using our somatic embryo-forming system, we identify a protein (called P-19.5 protein), which is preferentially accumulated during the early stage of cell clusters and globular embryos, and demonstrate that a cDNA clone encoding this P-19.5 protein shares a high degree of allergen protein that induces human allergy and is also regarded as a PR-like protein. Concomitantly, we find two separate cDNAs (*P-16* and *P-19.5 H* cDNAs) that their transcripts are separately expressed during somatic embryogenesis.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid

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2. Materials and methods

2.1. Preparation of cell clusters, globular embryos, heart-shaped, torpedo-shaped embryos, and regenerated plantlets

Regenerated plantlets were prepared as described in a previous paper [6]. In brief, surface-sterilized carrot seeds (*Daucus carota* L. cv Benishingsun) were germinated on solidified basal Murashige–Skoog (MS) [7] medium. Hypocotyl explants from seedlings were cultured in liquid MS medium with 1 mg/L of 2,4-D for 48 h, and then cultured in 2,4-D-free medium. After 3 weeks, cell clusters that had formed were transferred to the fresh basal MS medium without 2,4-D. These cell clusters regenerated plants within 2 weeks of transfer to the fresh medium. The procedure for somatic embryogenesis from regenerated plantlets was the same as that described previously [6]. Regenerated plantlets were cultured in MS medium containing 2,4-D for 24 h, and then transferred to 2,4-D-free medium. Subsequently somatic cells began to be released from whole regenerated plantlets at 2–3 days, and then to divide to form cell clusters at 3–4 days. Cell clusters (called 5-day cell cluster) were prepared by sieving the heterogeneous cell population from plantlets at 5 days with nylon screens of 117 and 41 μ m pore size. Similarly, 9-day cell cluster was prepared from plantlets cultured for 9 days with nylon screens of 297 and 117 μ m pore size. The cell clusters prepared from plantlets at 12 days with nylon screens of 297 and 84 μ m pore size were transferred to the fresh MS medium, and then continued to culture. After culture for 3–4 days, globular embryos were prepared by trapping the cell population with nylon screens of 200 μ m pore size. Subsequently, heart-shaped embryos were prepared from the cell population at 5–6 days and torpedo-shaped embryos, at 7–8 days. Finally, regenerated plantlets were prepared at 14 days.

2.2. Extracts of proteins and SDS-PAGE

Plant material at each stage from cell clusters to regenerated plantlets via embryos was homogenized in a Polytron homogenizer (Kinematica) for 2 min in two volumes of 10 mM phosphate buffer (pH 7.4) that contained 5 mM 2-mercaptoethanol and 5 mM MgSO_4 . The homogenate was centrifuged at 12,000 rpm for 30 min at 4 °C, and proteins in the supernatant fraction were precipitated by addition of solid ammonium sulfate to 80% saturation. Precipitated proteins were collected by centrifugation at 12,000 rpm for 20 min at 4 °C and dissolved in a small volume of 10 mM phosphate buffer (pH 7.4) that contained 5 mM 2-mercaptoethanol and 5 mM MgSO_4 . The resultant solution was dialyzed against the same buffer and applied to SDS-PAGE for analyzing the protein patterns during embryogenesis. In addition, the proteins fractionated by salting-out were purified to analyze N-terminus and internal peptide sequences of P-19.5, and to produce antibody of

P-19.5 for Western blotting, respectively. SDS-PAGE was performed with 15% (w/v) polyacrylamide gel using the buffer system of Laemmli [8]. Proteins were visualized by staining with Coomassie brilliant blue G-250.

2.3. N-terminus and internal peptide sequences of P-19.5 protein

Isolation of P-19.5 protein was carried out as follows. The protein solution partially purified by fractionation with ammonium sulfate as described above was applied to SDS-PAGE. After electrophoresis, both edges of the gel were excised, and then stained with Coomassie brilliant blue. The non-stained position corresponding to P-19.5 was excised, and P-19.5 protein was extracted from the excised gel in 70% formic acid.

The isolated P-19.5 protein was pyridylethylated by 4-vinylpyridine, and then subjected to in situ proteolytic digestion with lysyl endopeptidase in 0.5 M Tris–HCl (pH 7.5) containing 10 mM EDTA at 37 °C for 24 h. The released peptides were recovered from the supernatant and separated by reverse-phase HPLC. Purified peptide fractions were dotted on glass fiber membranes and subjected to automated Edman degradation on a sequencer. N-terminus sequence was identified as GVQKHEQEIT and four internal peptide sequences were identified as NVEIK, HITLPEGG-PVTMTL, IENNHL SVVPN, and AVEAYLIAN, respectively.

Isolation of P-16 protein and determination of its internal peptide sequence were carried out by the same procedure as P-19.5 protein.

2.4. Antibody production and Western blotting

In order to produce antibody of P-19.5 protein for Western blotting, P-19.5 protein that was partially purified by salting-out with ammonium sulfate, as described above was furthermore purified with DEAE-cellulose column chromatography, and then 2D-PAGE. The protein solution which were obtained by salting-out was dialyzed against 10 mM phosphate buffer (pH 7.4) that contained 5 mM 2-mercaptoethanol and 5 mM MgSO_4 , and then applied to DEAE-cellulose column (3 cm $\times$ 28 cm) that was pre-equilibrated with same buffer (data not shown). The proteins were eluted by a linear gradient of 0–0.3 M NaCl. Fractions containing P-19.5 were detected by SDS-PAGE and pooled. Pooled solution was dialyzed against deionized water, and then lyophilized. In addition, P-19.5 protein was purified by 2D-PAGE. 2D-PAGE was performed according to the O'Farrell's method [9]. In the first dimension, IEF extended from a mixture of pH 5–8 and pH 3.5–10, and 12.5% polyacrylamide-SDS gel slab was used in the second dimension. Gels were stained with Coomassie brilliant blue. P-19.5 protein was excised from the positions of P-19.5 on the gels, emulsified with Freund's incomplete adjuvant by Polytron homogenizer, and then injected to female New

Zealand White rabbits. Rabbits received injections 12 weeks after the initiation injection, and blood was drawn 2 weeks after the injection.

For Western blotting, proteins were electrophoretically transferred from SDS gel to PVDF membrane in 25 mM Tris, 192 mM glycine, 20% methanol, and 0.02% SDS. The membrane was soaked for 30 min in TBS-TS (0.15 M NaCl, 0.5% Triton X-100, 2 mM EDTA, 0.2% SDS, 2 mM Tris-HCl, pH 7.4) containing 2% skim milk to block non-specific binding sites at 4 °C overnight. P-19.5 protein antiserum (50 mL; 1:500 dilution) was then added and incubated for 60 min with gentle shaking. Following three more washes with TBS-TS, the proteins were visualized using peroxidase.

2.5. Cloning, DNA sequencing, and sequence analysis

Amplification, in a GeneAmp PCR system 2400 (Perkin-Elmer), involved 35 cycles at 94 °C for 30 s, at 55 °C for 1 min, and at 72 °C for 1 min. The last cycle was followed by a 7 min extension at 72 °C. The products of PCR were separated on a 1.0% low-melting-point agarose gel and stained with ethidium bromide. Then, bands of DNA fragments were cut from the gel, dissolved in TE buffer, and cDNA were purified with phenol-chloroform. The extracted DNA fragments were cloned into the *EcoRV* site of the Bluescript II SK + plasmid vector (Stratagene, La Jolla, CA). The cloned DNA fragments were sequenced with Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) and an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Computer-assisted searches for deduced amino acid sequences were carried out with the BLASTP sequences program [10].

2.6. Rapid amplification of 5' and 3' ends of cDNA (5'- and 3'-RACE)

The 5'- and 3'-RACE were achieved with a 5'-Full RACE Core Set and a 3'-Full RACE Core Set (Takara Shuzo, Kyoto, Japan) according to the manufacture's instructions.

2.7. Isolation of RNA and Northern blotting

Total RNA was isolated from 5- and 9-day cell clusters, globular embryos, heart-shaped and torpedo-shaped embryos, and regenerated plantlets using TRIzol reagent (Life Technologies) as described by the manufacture's published protocol for use with whole tissues.

Total RNA (5 µg per lane) were separated in electrophoresis in a formaldehyde gel containing 1.0% (w/v) agarose and transferred onto a Hybond Nylon membrane (Amersham) by standard methods. RNA was fixed by heating for 30 min at 120 °C and was hybridized with digoxigenin-labeled probes as described by the manufacturer (Boehringer Mannheim).

3. Results

3.1. Changes in protein pattern during somatic embryogenesis

To find the proteins that were specifically and preferentially accumulated at the early stage of carrot somatic embryogenesis, protein patterns of samples of each stage during embryogenesis were detected on SDS-polyacrylamide gel (Fig. 1A). A protein (called P-19.5 protein) with a molecular mass of 19.5 kDa was found to be preferentially present in significant amount at the early stage of 5- and 9-day cell clusters, and globular embryos, but drastically decreased in heart-shaped embryos and was hardly detected in torpedo-shaped embryos and regenerated plantlets. Western blotting revealed that the P-19.5 protein was preferentially accumulated at the early stage of cell clusters and globular embryos (Fig. 1B). However, in addition to P-19.5 protein, at least two bands corresponding to 18.6 kDa (P-18.6 protein) and 16 kDa (P-16 protein), respectively were recognized against the P-19.5 antiserum. Thus, these P-18.6 and P-16 proteins seem to have structural similarity as antigen with P-19.5 protein. P-18.6 protein was substantially similar to the P-19.5 protein with respect to the changeable pattern during somatic embryogenesis, whereas the P-16 protein was invariably present at all stages, as different from P-19.5 protein and P-18.6 protein.

3.2. Cloning and sequence of P-19.5 protein-encoding cDNA

Following homogenization of 9-day cell clusters, and then fractionation with ammonium sulfate precipitation, the P-19.5 protein was separated by SDS-PAGE, and then transferred to a PVDF membrane for N-terminal amino acid sequencing. N-terminus sequence of a protein band corresponding to P-19.5 protein on the membrane was identified as GVQKHEQEIT sequence by Edman degradation and was used to design a degenerate oligonucleotide for use in cloning. In addition, in order to ensure the design of a degenerate oligonucleotide for cloning and full-length sequence of this cDNA, we determined the amino acid sequences of internal peptides of the P-19.5 protein using a lysyl endopeptidase.

Antisense primers were designed according to the four peptide sequences obtained, and used for PCR in combination with a sense primer deduced from the N-terminal protein sequence. The P-19.5 full-length cDNA sequence was determined using 5'-RACE and 3'-RACE methods. The cDNA sequence of P-19.5 has a total length of 767 bases, excluding the poly (A)⁺ tail, and predicts an open reading frame of 154 amino acids (DDBJ accession no. AB082377). The ATG initiation codon started at nt 87 and the TAA termination codon started at nt 549. This cDNA sequence contained a continuous open reading frame comprising all five peptide sequences obtained directly from the P-19.5 protein. All

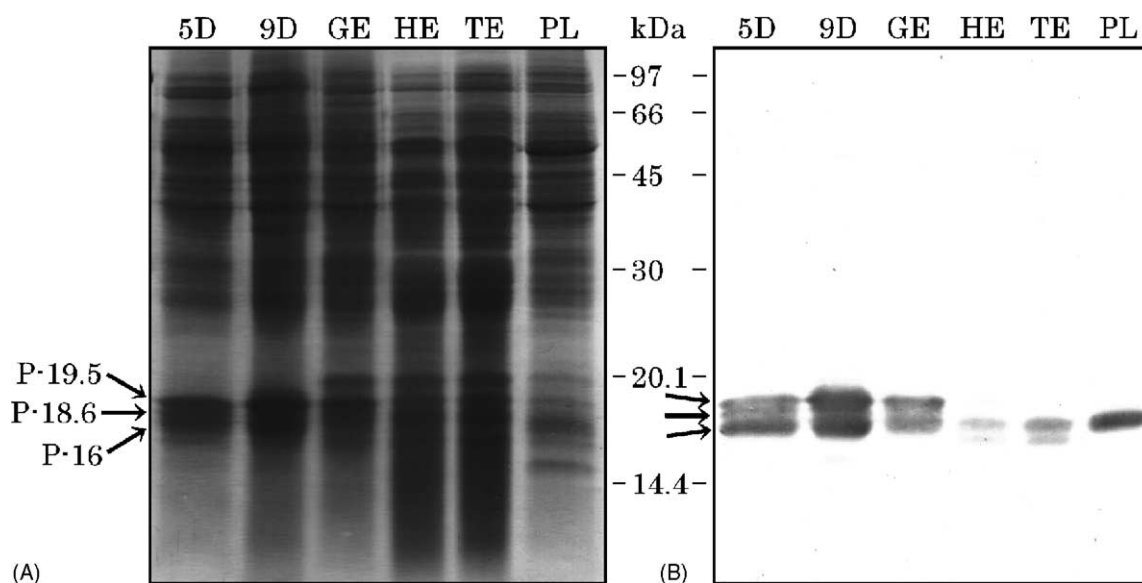


Fig. 1. Soluble proteins in somatic embryos of carrot at different stages of development. (A) Coomassie blue-stained SDS-PAGE gel. Proteins extracted from materials at each stage from cell clusters to regenerated plantlets were fractionated with ammonium sulfate, and then separated by SDS-PAGE. Each lane was loaded with 25 μ L of total protein extract. 5D, 5-day-old cell clusters; 9D, 9-day-old cell clusters; GE, globular embryos; HE, heart-shaped embryos; TE, torpedo-shaped embryos; and PL, regenerated plantlets. (B) Immune blot with antibodies against P-19.5. Proteins separated by SDS-PAGE were transferred to a PVDF membrane, and then analyzed with anti-P-19.5 antibody.

amino acids just adjacent to the N-terminuses of the four peptides that released by degradation with lysyl endopeptidase were lysine. Thus, the perfect identity of the five different peptide sequences independently obtained from P-19.5 protein indicates that *P-19.5* cDNA clone doubtlessly encodes the P-19.5 protein expressed preferentially at the early stage.

The molecular mass of predicted protein encoded by *P-19.5* was 16.6 kDa, which was less than 19.5 kDa estimated by SDS-PAGE. This difference was assumed from the result that P-19.5 protein appeared to be a glycoprotein because the predicted protein sequence contained a sugar-binding motif (Asn-X-Ser or Thr), and P-19.5 protein was actually recognized with concanavallin A lectin (data not shown).

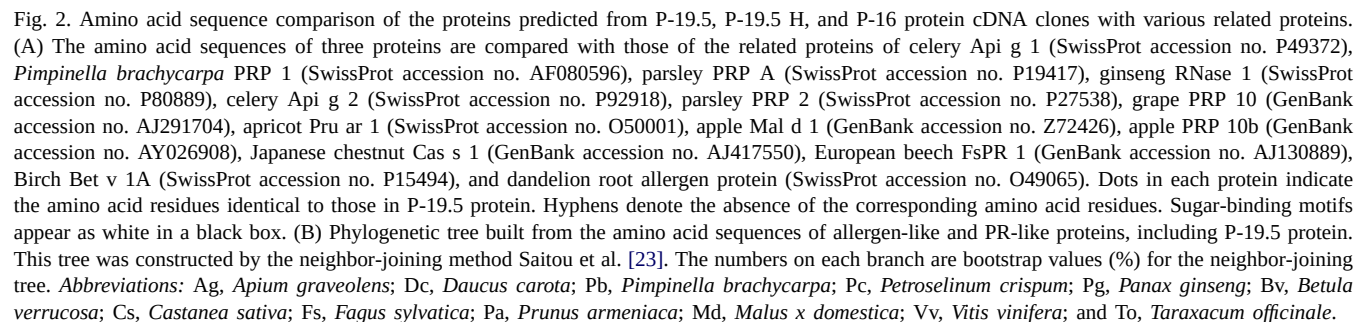
We found a separate cDNA that had highly homology with *P-19.5* cDNA clone during analyzing *P-19.5* cDNA with 5'-RACE and 3'-RACE methods. This full-length cDNA sequence had a total length of 730 bases, including the poly (A)⁺ tail, predicted an open reading frame of 154 amino acids (DDBJ accession no. AB127960). This amino acid sequence was 80.5% homology with that of *P-19.5* cDNA. We named this cDNA *P-19.5 homolog* cDNA (*P-19.5 H*). We anticipate that this *P-19.5 H* cDNA may probably encode P-18.6 protein because expression pattern of P-18.6 protein by Western blotting (Fig. 1) was highly agreed with that of the transcript of *P-19.5 H* cDNA, as described later.

On the other hand, isolation and cloning of P-16 protein that was present at all stages during somatic embryogenesis was also carried out with the similar procedure to that of P-19.5 protein. P-16 protein was isolated by SDS-electrophoresis from 9-day cell clusters, and then

four internal peptides were obtained from this isolated protein by the digestion with lysyl endopeptidase. Only the primer designed according to one peptide sequence (AAPGAYK) could be cloned among five peptides. The oligonucleotide sequence of this primer was also located in the *P-16* full-length cDNA sequence (DDBJ accession no. AB127961). Agarose gel electrophoresis of products with 3'-RACE revealed two bands. This fact indicates that one product had a sequence hydrolyzable with both *Xho*I and *Eco*RI and the other lacked such a sequence. In the *P-19.5* full-length cDNA clone, no sequence recognizable with two restriction enzymes was present. Thus, we anticipated that another band that was hydrolyzed with two enzymes might be a cDNA clone encoding the P-16 protein. The *P-16* cDNA encoded 154 amino acids and 70.1% amino acid identity to the P-19.5 protein.

Fig. 2A shows that there is homology in the open reading frame among the complete allergen protein sequences including P-19.5, P-19.5 H, and P-16 proteins. BLAST [10] database searches indicated that the amino acid sequence deduced from the nucleotide sequence of *P-19.5* cDNA clone shows a high degree of homology to celery major allergen Api g 1 protein (75%), carrot major allergen Dau c 1 group (70–72%), and the major birch (*Betula alba* L.) pollen allergen Bet v 1 (45%), which was a representative allergen. Thus, we regard the P-19.5 protein as an allergen-like protein. Furthermore, the cDNA sequence of this protein shared 71 and 63% identities with PR protein in *Pimpinella brachycarpa* and *Petroselinum crispum*, respectively.

It has already been found that carrot contains some allergens, that is, the Dau c 1 group is contained in the mature root and the green leaves [11], CR 16 in root [12], and



each stage during embryogenesis. Nearly full-length P-19.5 cDNA without the 5' non-coding region was used to obtain the random-primed probes. Simultaneously, the accumulation of *P-19.5 homolog* and *P-16* transcripts was examined by the same method of *P-19.5*.

As shown in Fig. 3, the *P-19.5* transcript was observed to be highly expressed in the cell clusters at the early and late stage, and in globular embryos. Subsequently, the expression of *P-19.5* gene was drastically decreased in heart-shaped embryos and detected very slightly in torpedo-shaped embryos and plantlets. This expression pattern of the *P-19.5* transcript during somatic embryogenesis was in fair agreement with that of *P-19.5* protein assayed by Western blotting (Fig. 1). *P-19.5 H* gene was highly expressed in the cell clusters and subsequently, was drastically decreased in globular embryos, though hardly detected in heart- and torpedo-shaped embryos. The expression pattern of *P-19.5 H* gene was agreed with that of *P-18.6* protein by Western blotting. Compared to *P-19.5* gene, *P-19.5 H* gene was expressed in cell clusters at an earlier stage during somatic embryogenesis. *P-16* gene was, however, invariably expressed at all stages from cell clusters to plantlets. Furthermore, this expression pattern highly agreed with that of *P-16* protein.

Northern blotting assay was used to assess the accumulation of *P-19.5*, transcripts in cell clusters and embryos at

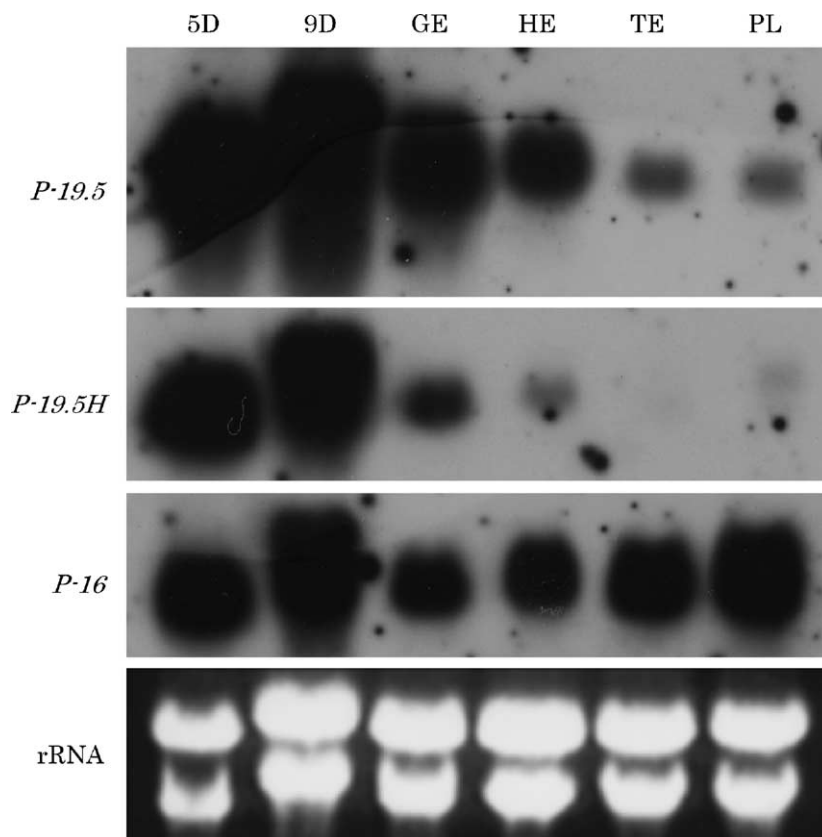


Fig. 3. Northern blotting analysis of *P-19.5*, *P-19.5 H*, and *P-16* gene expressions at six stages of embryogenesis. Total RNAs were isolated from 5-day-old cell clusters (5D), 9-day-old cell clusters (9D), globular embryos (GE), heart-shaped embryos (HE), torpedo-shaped embryos (TE), and regenerated plantlets (PL), and each RNA was loaded at 5 μ g per lane.

4. Discussion

In the present study, we characterized a cDNA clone encoding P-19.5 protein as an allergen-like protein that is preferentially accumulated at the earliest stage of cell clusters and globular embryos during carrot somatic embryogenesis, and we simultaneously examined *P-16* cDNA and a separate cDNA (*P-19.5 H*) different from *P-19.5* and *P-16* cDNAs that was inadvertently found when analyzing the full-length cDNA sequence of *P-19.5*. Although allergen proteins have been identified in various fruits and vegetables such as apple, celery, cherry, carrot, and potato, little is known about the fundamental function of allergens in plants. However, they have been assumed to play some role based on their similarity to known proteins.

The major birch pollen allergen, Bet v 1, has been shown to be homologous to PR proteins in a number of plants. Moreover, the protein encoded by *P-19.5* cDNA shared 45% identity with Bet v 1, and 71 and 63% identities with PR proteins from *Pimpinella brachycarpa* and *Petroselinum crispum*, suggesting that P-19.5 protein as well as other allergen proteins can be considered PR-like proteins. Similarly, P-19.5 H and P-16 proteins as well as P-19.5 protein can be regarded as PR-like proteins. PR proteins have so

far been found to not only play a role in the defense system against pathogen infection [14–17], but also to have functions related in an organ- and tissue-specific manner during development programs such as the germination, senescence, and flowering of healthy plants [2,4,18]. Simultaneously, those PR-like proteins may be involved in sequence events in somatic embryogenesis as part of the development functions of healthy plants. Although to date there has been no actual evidence for the involvement of PR-like proteins in somatic embryogenesis, there are an increasing number of reports on PR-like proteins that suggest such an involvement, including the P-19.5 protein presented in this study. Thus, Lin et al. [13] reported that, in the development from globular embryos to regenerated plantlets during carrot embryogenesis, there are two PR-like proteins, Gea 20 expressed in plantlets and seedlings, and Gea 31 expressed at stages from globular embryos to plantlets. We reported previously that in our somatic embryo-forming system, the transcript of no. 43 protein, a thaumatin-like protein with a high homology to PR protein, was preferentially expressed at the earliest stage of cell clusters formed by the division of somatic cells [1]. P-19.5 proteins as well as no. 43 (thaumatin-like protein) seem to be associated with the induction and initiation of embryogenic cell clusters from

carrot somatic cells, since the transcripts of their genes were preferentially expressed at the earliest stage. P-16 protein found concomitantly with P-19.5 protein was present at all stages during somatic embryogenesis. Similarly, Gea 20 and Gea 31 found by Lin et al. [13] were preferentially accumulated in plantlets and seedling at stages from globular embryo to plantlet, respectively. Thus, in carrot embryogenesis, it is of interest to note that there may be some kinds of PR-like proteins having specific functions for sequential events from the early stage when somatic cells divide to form cell clusters to the late stage of plantlets [1].

Helleboid et al. [5] reported that the direct somatic embryogenesis process in *Cichorium* is accompanied by an increase in the amounts of PR proteins, osmotin-like protein, chitinase, and β -1,3-glucanase. It is noteworthy that PR-like proteins such as thaumatin, allergen, osmotin, and hydrolytic enzymes such as chitinase and β -1,3-glucanase share a characteristic common to somatic embryogenic cultures. No P-19.5 protein has the typical signal sequence of proteins targeting some organelle and extracellular medium, suggesting that it locates predominantly in the cytosol. In addition, PR proteins in *Pimpinella brachycarpa* and *Petroselinum crispum*, which are homologous to the P-19.5 protein gene also have no signal sequence. During the somatic embryogenesis of *Cichorium*, PR proteins such as osmotin-like protein, chitinase, and β -1,3-glucanase were accumulated in the medium of embryo cultures at a much higher level when compared to their level in the medium of a non-embryogenic cultures. Thus, PR-like proteins that seem to be involved in somatic embryogenesis may be of two different intracellular and extracellular types.

On the other hand, allergens of the major birch pollen and ginseng show ribonuclease activity [19], as do profilins that regulate actin polymerization and participate in signal transduction by way of the phosphoinositide-signaling pathway [20]. Allergens of grass pollen are acknowledged to be structurally and functionally related to the expansins, which induce extension of plant cell walls [21]. In addition, allergens from brassica appear to have a similarity to calmodulin that binds calcium [22]. Thus, high-sequence conservation among homologies in a number of plants suggests that allergen proteins serve a vital biological function.

Although the PR-like protein genes identified here may be closely related to the induction of carrot somatic embryos, relevant direct evidence can be derived from experiments in which the antisense or over-expression transformation of genes results in the suppression or induction of embryogenesis.

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