

Short Communication

## A Major Root Protein of Carrots with High Homology to Intracellular Pathogenesis-Related (PR) Proteins and Pollen Allergens

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We isolated the cDNA clone coding for a major root specific protein (CR16) of carrots. The CR16 protein (154a.a.) has a high homology to intracellular pathogenesis-related (PR) proteins, stress-induced proteins and also the major allergen protein of celery (Api g1). The CR16 protein gene formed a super gene family and transcripts of this CR16 protein gene are predominant in root tissue, not in leaves or flowers.

**Key words:** cDNA cloning — *Daucus carota* — Gene family — Organ-specific expression — Plant defense mechanism — Pollen allergen.

The root is of a fairly simple architecture consisting of epidermis, cortex, endodermis and stele and basic structure of the root tissue is fairly common in all species of plants (Esau 1940). However, functions of each specialized fine structure of root tissue and their protein components are less well characterized in comparison with those of leaf, stem and flower. Several root specific cDNAs and genes have been cloned from roots of various plants. The barley lectin gene (BLc3) was isolated from a barley embryo and the transcript was found in both embryo and root tips (Lerner and Raikhel 1989). Extensins and P33, which are hydroxyproline-rich glycoproteins were also identified as cell wall proteins in roots and the transcripts increased markedly after wounding (Tierney et al. 1988). Furthermore, a root-specific protein (TobRB7) with high homology to a water channel protein has been cloned from tobacco plants (Yamamoto et al. 1990). Thus, molecular cloning is useful tool to identify protein components in roots. Differential screening is an efficient method to isolate cDNA clones exhibiting root-specific expression. Biochemical analysis of proteins is an alternative way to identify root specific proteins. Protein profiles such as molecular weight, pI value and location can be characterized during experi-

mental processes of protein analysis. Biochemical analysis of protein components in roots has been limited. Compared with aerial parts of plant such as leaves, stems and flowers, the roots contain higher amounts of water and reversibly low levels of proteins, an event presenting a major obstacle to characterize protein components in root tissue. However, protein components of root tissues could be resolved when we used ten to twenty-fold concentrated crude extracts for the protein analysis. We, therefore, tried to perform a direct protein analysis of carrot roots.

As the first step to characterize protein components of root tissue, we prepared soluble extracts of mature roots of carrots (*Daucus carota*) varieties, *Kuroda-gosun* and soluble proteins (1 µg) was separated by SDS-PAGE (Laemmli 1970). Consequently, three major protein bands, with a molecular mass of 41, 40 and 16 kDa, respectively, appeared, and each was estimated to be about 10% of the total protein. Next, the variety *Kuroda-gosun* was seeded and grown in soil pots, and plant materials were collected at several different stages of development. At seven, nine, and twenty-one weeks (mature stage) after seeding, roots as well as aerial parts were collected separately. As carrot seedling at four weeks was extremely small, the total seedlings with both roots and aerial parts were used to determine protein compositions. As seen in roots of mature carrots, the protein concentration of root tissues from all the samples was about ten to twenty-fold lower than in leaves and stems. When compared with the protein band patterns, one of three major proteins, the 16 kDa one was the only protein whose expression pattern was root-specific (Fig. 1a). The 16 kDa protein (CR16) was not observed in 4 weeks seedlings yet was the most distinct protein in root tissues at later stages of development. No other evident root-specific protein bands were found in this experiment. The relative content increased as the carrot plants were developing to mature forms. On the contrary, two other proteins, 41 and 40 kDa proteins were found in leaves and stems as well as in roots. We have no evidence that 41 and 40 kDa proteins in aerial parts are identical ones in roots but their contents were fairly constant through all stages of development. Although contents of many other proteins decrease during development, the content of the CR16 protein of root increased. The *Kuroda-gosun* is one representative variety in Japan but many other varieties are also available, all of

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PR, pathogenesis-related; PVDF, polyvinylpyrrolidone; ss, single-strand.

The nucleotide sequence reported in this paper has been submitted to DDBJ/EMBL/Gene bank with accession number D 88388.

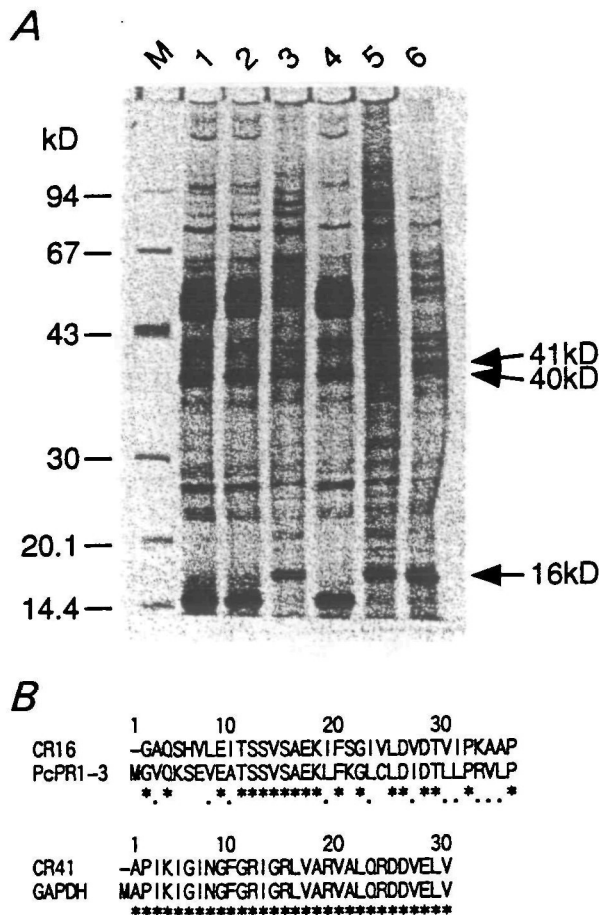


**Fig. 1** Protein analysis of carrot root and leaf at different stages of development. (a) Roots, stems and leaves collected from carrot plants were placed in a mortar and liquid nitrogen was added. The frozen samples were broken by a wood hammer and the broken sample was crushed in a Waring blender (Nikon-Seiki) at 1,500 rpm for 25 min to obtain a powder sample. 12.5 ml of protein extraction buffer (0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, 0.1% ascorbate, 0.28% 2-mercaptoethanol) was added to this sample which was then placed in a Waring blender at 2,000 rpm for 5 min. The obtained preparation was passed through four layers of gauze and the filtrate was centrifuged by 10,000 rpm for 10 minutes at 4°C to obtain the soluble fraction. The resulting filtrate was concentrated by Amicon ultrafree-CL unit (Millipore) up to about 2 mg ml<sup>-1</sup> of protein concentration. The concentrated soluble fractions from root tissues and non-concentrated fractions from leaf tissues were analyzed on 10% SDS-PAGE. The separated protein bands were visualized following silver staining. Lanes; 1, four weeks seedlings; 2, seven weeks leaf; 3, seven weeks root; 4, nine weeks leaf; 5, nine weeks root; 6, mature plant root. (b) Homology search of the carrot 16 kDa and 41 kDa proteins. The amino terminal sequences of 41 kDa, 40 kDa and 16 kDa were determined using a protein sequencer. Homology search data for CR16 and the 40 kDa protein were also indicated.

which can be classified into four or five subgroups according to RAPD analysis (Nakajima et al. 1997). To determine if CR16 protein is present in other types of carrots, we next analyzed root proteins from three other varieties, *Early Chantenay*, *Imperator* and *Nantes Scarlet*. Consequently, all these varieties also contained the 16 kDa protein in the root tissue as found in *Kuroda-gosun*. Expression levels and patterns were much the same as found in *Kuroda-gosun* (data not shown). After soluble proteins of carrot roots were separated on 10% SDS-PAGE, protein bands were transferred to a PVDF membrane and silver stained. Protein bands corresponding to the 41 kDa, 40 kDa and 16 kDa proteins were cut out from the gel, protein samples (300 pmol) were extracted from the gels and amino acid sequences were determined using a sequencer (ABI 473A, Applied Biosystems (ABI), Foster City). Thirty-five amino terminal residues of the CR16 protein were determined (Fig. 1b). Within this amino acid sequence, no unique motif was observed but five serine residues were present within twenty amino acid residues. Interestingly, the obtained amino-terminal sequence has high homology to parsley pathogenesis-related (PR) protein, strongly induced by viruses and pathogens (Schmelzer et al. 1989, Somssich et al. 1988). On the contrary, thirty and twenty-eight amino-terminal residues of the 41 and 40 kDa proteins were determined, respectively. Unexpectedly, two sequences were exactly the same. The obtained sequence has perfect homology to the amino-terminal sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the snapdragon (Fig. 1b). The GAPDH plays a central role for glycolysis in plants and expression is thought to be constitutive in all tissues (Shih et al. 1991). There are three known types of GAPDH genes named chloroplastic GapA, GapB and cytosolic GapC. The 41 and 40 kDa proteins might belong to the cytosolic type and cytosolic GAPDH expression is linked to light as well as the metabolic state (Shih et al. 1991). However, precise function of this protein in metabolic regulation remains unclear. Several proteins from carrot roots have been purified and characterized. A fructose-1,6 bis phosphate aldolase (Moorhead and Plaxton 1990), carotenoprotein (Bryant et al. 1992), DcDrP1 protein (Ebener et al. 1993), and 6-hydroxymellein synthetase (Kurosaki et al. 1991) were isolated and contents of these proteins in carrot roots were reported to be relatively high. However, no common features were found between the CR16 and these proteins. The CR16 protein was the only major root-specific protein detected in this analysis, therefore, further studies focused on the CR16 protein.

To isolate the cDNA clone coding for the CR16 protein, a lambda phage library was constructed from the poly(A)<sup>+</sup> RNA of carrot root tissues. We next designed two PCR primers for amplification of the specific DNA band, which corresponds to the amino-terminal segment of CR16 protein. RT-PCR experiments were performed using 1 µl

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      10      20      30      40      50      60
CATTCTAAATATCATGGGTGCCAGACCATTCAGATCACTTCTTCAGTCTCCGC
      M G A Q S H S L E I T S S V S A

      70      80      90      100     110     120
AGAGAAAATATTCAGCGGCATTGTCCTTGATGTTGATACAGTTATCCCAAGGCTGCCCC
      E K I F S G I V L D V D T V I P K A A P

      130     140     150     160     170     180
CGGAGCTTACAAGAGTGTGATGTTAAAGGAGACGGTGGAGCTGGAACCGTCAGAATTAT
      G A Y K S V D V K G D G G A G T V R I I

      190     200     210     220     230     240
CACCCCTTCCCGAAGGTAGCCCAATCACCTCAATGACGGTTAGGACTGATGCAGTGAACAA
      T L P E G S P I T S M T V R T D A V N K

      250     260     270     280     290     300
GGAGGCCTTGACATACGATTCCACAGTCATTGATGGAGACATCCTTCTAGAATTCATCGA
      E A L T Y D S T V I D G D I L L E F I E

      310     320     330     340     350     360
ATCCATTGAAACCCATATGGTAGTTGTGCCAACTGCTGACGGAGGTAGCATTACCAAGAC
      S I E T H M V V V P T A D G G S I T K T

      370     380     390     400     410     420
CACTGCCATATTCCACACCAAAGGCGATGCCGTGGTTCCTGAGGAGAACATCAAGTTTGC
      T A I F H T K G D A V V P E E N I K F A

      430     440     450     460     470     480
AGATGCTCAGAACACTGCTCTTTTCAAGGCTATTGAGGCCTACCTCATTGCTAATTAAGC
      D A Q N T A L F K A I E A Y L I A N *

      490     500     510     520     530     540
TGAGCTCTCAACTTCCGTAATTTTATGAGTGAGTGGAGGAATTGCAACGTTTCTTTTGT

      550     560     570     580     590     600
GTTTTGTTTTCGAGCAACTTCATAATTTACAGAGTGAGTGACAGTCAGTGACAGAATTGC

      610     620     630     640     650     660
AACTTTCTCTTTGTACTTTGTTGTGACTTGTGATGAATAACTTCATCTGGCTGGTAATGT

      670     680     690     700     710     720
ATGCGATCTTTTTAAATAATATGCACTATTATTAACCAATAATCATATTCTTCTCAA

      730     739
AAAAAAAAAAAAAAAAAAAA

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**Fig. 2** The entire nucleotide sequence of the CR16 protein cDNA. The full-length cDNA clone coding for the CR16 protein was isolated from the lambda recombinant library and the nucleotide sequence determined. Ten g of root tissue (fresh weight) of carrot, harvested at 9 weeks after seeding was placed in 10 ml of liquid nitrogen. The frozen tissue was broken up using a wood hammer and then completely crushed in a Waring blender at 1,500 rpm for 25 min to obtain the powder sample. From the resulting powder sample total RNA was prepared using RNA isolation kits (Clontech, California). Poly(A)<sup>+</sup> mRNA was purified using oligo(dT) cellulose chromatography, done following description in the manual from the supplier. cDNA synthesis was performed from 1 µg of poly(A)<sup>+</sup> mRNA, according to the supplier's manual (Amersham, Japan). The obtained carrot root cDNA was ligated into the lambda ZAPII vector (Stratagene, U.S.A.) and the recombinant molecules were packaged into lambda phage particles to construct the recombinant phage library. The amplified RT-PCR fragment was used as a probe to screen the CR16 cDNA clone. Preparation of <sup>32</sup>P-labeled probe and phage library screening were carried out according to Maniatis et al. (1982). Hybridization was carried out overnight with hybridization buffer (5 × Denhardt's, 5 × SSPE, 0.1% SDS, 100 µg ml<sup>-1</sup> ssDNA), at 45°C. The membrane was washed twice in 6 × SSC at room temperature for 5 min, then 6 × SSC, 0.1% SDS at 45°C for 10 min. Hybridization bands were visualized using an Image analyzer (BAS2000; Fuji Film, Tokyo). The cDNA coding for CR16 protein was subcloned into the pBluescript SK(−) vector from the isolated lambda phage. Sequencing was also done as described by Maniatis et al. (1982). The open reading frame (465 nucleotides) encodes 154 amino acids, and the molecular mass is estimated to be 16,129.04 Da.

was identical to that from the data on protein sequencing experiments. However, the seventh amino terminal residue within 35 amino acid residues was serine instead of valine in case of the CR16 cDNA clone. Typical signal sequences of protein targeting for chloroplast, mitochondria, vacuole or endoplasmic reticulum were not observed, which means that the CR16 protein locates predominantly in the cytosol fraction. Detailed histochemical analysis using antibodies against the CR16 protein might reveal location of the CR16 protein.

Various intracellular PR protein genes which are highly homologous to the CR16 root-specific protein gene have been isolated from parsley, bean, potato and asparagus (Schmelzer et al. 1989, Somssich et al. 1988, Walter et al. 1990, Constabel and Brisson 1992, Warner et al. 1992). These intracellular PR proteins have no signal sequences as seen in the CR16 protein. A phylogenetic tree was constructed based on the weighted pair-group method with arithmetic averages (UPGMA). Consequently, all the CR16 related proteins so far reported could be classified into four groups (data not shown). The CR16 protein

|                   |                                                                                           |     |
|-------------------|-------------------------------------------------------------------------------------------|-----|
| CR16C1 (Carrot)   | 97: SIETHMMVPTADGGSITKTTAIFHTKGDAAVPEENIKFADAQNTALFKAIEAYLIAN                             | 154 |
| Api1g1 (Celery)   | 97: . . . N. V. L. . . . . C. . . . . Y. NE. . . . . L. . . . .                           | 154 |
| PcPR1-3 (Parsley) | 98: . . NN. FTA. N. . . CTV. S. I. . N. . . . . ND. LT. . . V. . . . .                    | 155 |
| Betv1 (Birch)     | 98: K. SNEIKI. A. P. . . . L. ISNKY. . . . . HE. KA. QV. ASKEMAGET. LR. V. S. . L. HSDAYN | 160 |
| Cora1 (Hazel)     | 98: KVCHELKI. AAPG. . . . L. ISSK. . A. . . HEINA. EML. G. KEMAEK. LR. V. T. L. HSAEYN    | 160 |
| Mald1 (Apple)     | 98: K. SYETKL. AAGS. ST. . . . SISHY. . . . NIEIK. . HV. VGKEKAH. G. . . . L. S. KDHPDAYN | 159 |
| STH-2 (Potato)    | 93: . . SYDLKFEAHGN. CVC. SITEY. . . . Y. LKD. EHNEGOK. GME. . . IV. . . . L. PSVYA       | 155 |
| PvPR1 (Bean)      | 98: K. TFD SKLS DGP. . . LI. LSITY. S. . . . PPN. DEL. AGK. KSDS. . . V. . . . L. P       | 156 |
| SAM22 (Soybean)   | 97: K. TFD SKL. AGPN. . . AG. L. VKYE. . . . EPNDEL. TKG. KAD. . . . L. HP-DYN            | 158 |
| P149 (Pea)        | 97: K. SFEAKLSAGPN. . . A. LSVKYF. . . . APS. : QL. TDK. GGDG. . . L. G. CL. HP-DYN       | 158 |
| ABR17 (Pea)       | 97: KVAFETIILAGS. . . . V. ISVKY. . . . . ALSDAVRDETK. KG. G. I. . . . G. -VLANPGY-       | 157 |
| AoPR1 (Asparagus) | 99: CAT. . . FKFE. SSN. . CLV. V. . SYKILPGVADESAKA. EGITN-H-M. . . T. . . L. PTAYV       | 158 |
| PBZ1 (Rice)       | 96: . HS. ETKLEA. G. . SCVA. LKVEYELEDGSSLSLP. KE. DIVDGYGML. M. . . D. . V. HPAEYA       | 158 |

**Fig. 3** Highly homologous regions of the carrot CR16 protein to various proteins. The amino acid sequence of the CR16 protein is compared with the related proteins of celery AP1g1, parsley PcPR1-3, birch BetV1 allergen, hazal Coral allergen, apple Mald1 allergen, potato STH, bean PvPr1, soybean SAM22, pea PI49, pea ABR17, asparagus AoPR1, and rice PBZ1. Dots in each protein indicate the identical amino acid residues to that in the CR16 protein. Hyphens show the absence of the corresponding amino acid residues after homology match analysis. Asterisks indicate amino acids conserved among all thirteen sequences.

belongs to the Group I involving celery Apig1 (Breitender et al. 1995) as well as parsley PR1-1 and 1-3 proteins (Schmelzer et al. 1989, Somssich et al. 1988). One of the most highly homologous proteins to the CR16 protein was celery Apig1 (Breitender et al. 1995). The CR16-related proteins were isolated from various plant materials such as fruit, leaf, pollen and suspension cultures but the CR16 protein is the intracellular PR protein to be isolated from roots. The CR16-related intracellular proteins of *Tagetes patula* also have been isolated from roots although their reported amino acid sequences are not full-size (Jacob et al. 1995). In alignment of ten CR16 related protein sequences, seventeen residues are common among these proteins and these common residues are randomly located within the proteins (Fig. 3). Glycine residues are frequent but we have no conclusive explanation for these conserved amino acid residues. Protein domain analysis also indicated that two possible phosphorylation sites are involved in the CR16 protein, the putative phosphorylation sites are located at the amino terminal half (52(TVR)54 and 68(TVR)70). The calculated pI of the CR16 was estimated to be 4.4. Other closely related proteins also exhibit the acidic pI value (Walter et al. 1990).

As observed in other related protein genes, the CR16 protein gene is expected to form a supergene family. Southern hybridization experiments were done with the CR16 protein cDNA as a probe for the *EcoRI*, *BamHI*, *HindIII* and *XbaI* digested DNA fragments. Many positive signals were obtained with all digested DNA, under highly stringent conditions for washing (Fig. 4a). Several related genes for the CR16 protein might exist in the carrot genome. Northern blotting experiments were done in which the specific probe for the 3'-nontranslated region of the CR16 protein cDNA was used for analysis of mRNAs from root, leaf and flower. As expected, messages for the CR16 protein gene predominantly accumulated only in root tissues (Fig. 4b). Size of the mRNA was consistent with that of the calculated message from the cloned CR16 cDNA.

Pathogen infection induces many intracellular PR proteins with a high homology to CR16 proteins. In potato tuber, the defense-related STH-2 protein is rapidly induced by infection of *Phytophthora infestans* zoospores (Constabel et al. 1992). The *Asparagus officinalis* intracellular PR1 (AoPR1) gene is also expressed in response to pathogen attack (Warner et al. 1993). Elicitor treatments also induce intracellular-PR proteins with high homology to CR16 protein in peas, beans and soybeans (Fristensky et al. 1988, Chiang and Hedwiger 1990, Walter et al. 1990). We, therefore, plan to do the pathogen infection tests and elicitor treatment experiments with carrot roots to determine if CR16 gene expression is induced by pathogens. Cellular signal compounds such as arachidonic acid and salicylic acid induced PR10a (formerly STH2) (Matton et al. 1990) and AoPR1 gene expression (Warner et al. 1994, Mur et al.

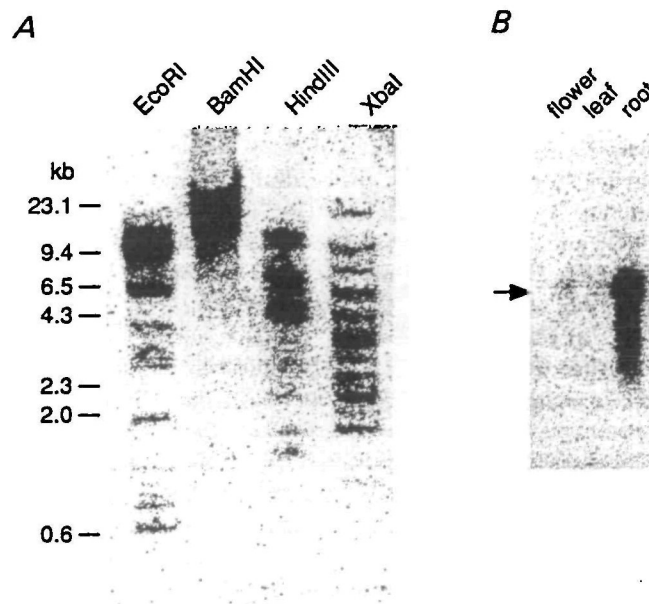


Fig. 4 Gene family and expression pattern of the CR16 protein gene. (a) Ten  $\mu$ g of DNA isolated from carrot leaves were digested with *EcoRI*, *BamHI* and *XbaI*. Carrot genomic DNA was prepared as described (Nakajima et al. 1997). Digested carrot DNA samples were then fractionated by 0.8% (w/v) agarose gel electrophoresis, treated with 0.25 M HCl for 10 min, denatured in 1.5 M NaCl/0.5 M NaOH for 30 min, blotted onto nylon membrane Hybond N (Amersham, Japan) and annealed to DNA probes as described (Maniatis et al. 1982). The DNA probe was prepared from the entire cDNA fragment of CR16 clone. (b) Expression pattern of the CR16 protein gene in carrot. For Northern analysis, total RNA was isolated from carrot flower, leaf and root, and poly(A)<sup>+</sup> RNA were purified using oligotex-dT30 (Takara shuzo, Kyoto). Two  $\mu$ g of poly(A)<sup>+</sup> RNA in each lane were separated on 1% agarose gels containing formaldehyde according to Maniatis et al. (Maniatis et al. 1982). The obtained gels were blotted onto nylon membrane and hybridized with the 3'-noncoding region of the CR16 protein gene as a probe.

1996), respectively. Furthermore, the chemically synthesized compounds, probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide) (Midoh and Iwata 1996), 2,6-dichloroisonicotinic acid and benzo (1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Gorlach et al. 1996) also induced acquired disease resistance in plants and one of the probenazole induced proteins was identified as intracellular PR protein. These agrochemicals induce resistance to disease in a variety of plants including monocot and dicot, suggesting that a common signal pathway for establishment of the disease resistance is evolutionally conserved. These signal compounds might also function as cellular signals for enhanced gene expression of CR16 protein gene.

The major pollen allergen BetV1 from white birch, one of the main causes of Type I allergic reactions is highly homologous to several intracellular PR proteins, including CR16 protein (Breitender et al. 1989, Swoboda et al.

1994). BetV1 displays a considerable degree of heterogeneity, and thirteen different cDNA clones coding for BetV1 isomers were obtained by polymerase chain reaction amplification of birch pollen cDNA (Swoboda et al. 1995). Thus, formation of the multigene family is thought to be a common feature of intracellular PR protein genes. Based on available data carrot pollens might contain CR16 related proteins. Although the enzyme activity of the intracellular PR proteins remains unclear, intracellular PR proteins from parsley have a high sequence homology to RNase from ginseng calluses (Moseyev et al. 1994). Therefore, intracellular PR proteins expressed in pollen may be involved in recognition processes between different species. In case of pathogen-host interaction, CR16 protein may play an important role to degrade pathogen specific RNAs.

Southern blots revealed that the CR16 related genes form a super gene family. Each intracellular PR protein may well have individual biological functions. We are currently cloning and characterizing the promoter region of the CR16 protein genes to find regulatory mechanisms of CR16 gene expression.

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