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Research Note

Studies on the Response of Carrot Cells to a *Sclerotinia sclerotiorum* Elicitor: Induction of the Expression of an Extracellular Glycoprotein mRNA

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A heat-released elicitor (HRE) obtained from *Sclerotinia sclerotiorum* mycelium induced a transient increase in the activity of phenylalanine-ammonia lyase (PAL) in a carrot cell culture. Differential display reactions carried out on total RNA extracted from cells exposed for 7 h to the HRE revealed a complex cell response involving the induction and repression of several mRNAs. One of these elicitor-induced mRNAs encodes a 42.6-kDa protein. Northern (RNA) blot analysis and reverse-transcription polymerase chain reaction studies showed that noninduced cells have a basal expression of the 42.6-kDa protein mRNA that was stimulated five- to 10-fold by treatment with the elicitor.

Additional keyword: plant defense.

Most plants are resistant to most pathogens; nevertheless, if the environment is conducive and a virulent pathogen is present, defense mechanisms are necessary to prevent disease (Keen 1990). An inducible response that often correlates with resistance is the hypersensitive reaction (HR), characterized by plant cell death at the site of infection of an invading pathogen. Although the molecular mechanisms that lead to limitation of pathogen growth are obscure, it is thought that the HR plays an important role (Staskawicz et al. 1995). In some fungal infections, plant defenses can be activated by elicitors derived from microbial cell fluids or walls (Messiaen and Van Cutsem 1993; Bowles 1990; Lamb et al. 1989; Ryan 1988).

A systematic search for genes that are transcriptionally activated or inactivated by exposure of cells to elicitors may provide a better understanding of the complex plant response to pathogens at the molecular level. This might be helpful for pathogens, such as *Sclerotinia sclerotiorum* (Lib.) de Bary, that produce diseases that are difficult to control by traditional methods. This fungus causes severe disease in a wide variety of cultivated plants and no resistant hybrids or cultivars have been obtained so far. We have employed carrot cell cultures to identify differentially expressed plant genes responsive to a new *S. sclerotiorum* cell wall–derived elicitor. Partial characterization and purification of this elicitor showed that it is a mannose containing polysaccharide (C. Bertinetti and R. A. Ugalde, manuscript in preparation).

In this report we describe the molecular cloning of a novel elicitor-induced gene from carrot, employing a methodology not explored so far to search for possible resistance genes in sclerotiniosis. It consists of looking for induced genes in susceptible carrot cell cultures exposed to a mycelium heat-released elicitor (HRE) extract by the differential display polymerase chain reaction method described by Liang and Pardee (1992). The strategy is to isolate inducible genes that might be introduced under strong and constitutive promoters into model plants to be evaluated for their potential role as resistance genes.

Carrot cell cultures treated with a final concentration of 100 µg of glucose equivalent of HRE per ml (obtained according to the method of Ayers et al. [1976]) showed a rapid increase in the activity of phenylalanine-ammonia lyase (PAL) (data not shown). The kinetics of the induction suggested that the increase in PAL activity was due to gene induction, as has been described for some plant-pathogen interactions (Lawton and Lamb 1987). Accordingly, the isolation of activated genes under these conditions might be useful not only for the characterization of this plant-pathogen interaction but also for the identification and isolation of potential resistance genes.

Total RNA was obtained from 7 h HRE-treated carrot cells and compared by the differential display method with total RNA from untreated control cells. In brief, total RNA from carrot cells was prepared as described by Chomczynski and Sacchi (1987), and treated with RNase-free DNase (Promega, Madison, WI) according to the manufacturer's instructions to remove DNA contamination. Reverse transcription was carried out as described by Maniatis et al. (1982), with the oligonucleotide 5′-T_{11}GC-3′ as primer. PCR reactions were carried out with primer 5′-T_{11}GC-3′ and the arbitrary 10-base primer 5′-CGGCCCCTGT-3′ (OLI-5) in the presence of 0.5 µM ^{35}S-labeled dATP and 2 µM dNTPs. PCR reaction conditions were as described previously (Liang and Pardee 1992).

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Three amplified DNA fragments of approximately 300, 200, and 120 bp clearly differentiated control from treated cells (Fig. 1). This pattern of amplified products was reproducibly obtained in independent PCR reactions and with RNA prepared from independently treated cultures (data not shown). The putative induced amplified cDNAs were excised from the gel, eluted with water, re-amplified by PCR, and cloned. Clone pCMB14, containing the 300-bp product indicated by an arrow in Figure 1, was further characterized. Sequence analysis revealed that pCMB14 contained a 308-bp insert (Fig. 2, position 1853 to 2161), with an open reading frame (ORF) of 56 amino acids. The consensus polyadenylation signal sequence (AATAAA), which is often found 18 to 36 nucleotides upstream of the poly (A) site of plant genes (Joshi 1987), was observed. A GT rich box around 20 bp upstream of the poly (A) signal required for its efficient function (Wu et al. 1995) was also present (Fig. 2). These results revealed that this amplified product was generated by an mRNA that is present in HRE-induced cells and absent, or present at very low concentration, in control cells; experiments to discern between these two alternatives were carried out.

Based on the nucleotide sequence of pCMB14, oligonucleotides DCC 20 (5′-CCGTTTATTTGTAATATCCTCTTA-3′) and DCC 21 (5′-TAATATCCTCTTACAACAACAGAT-3′) were designed to carry out a 5′ RACE (rapid amplification of cDNA ends) reaction in order to obtain the full-length cDNA, with a commercial kit used according to the manufacturer's instructions (GIBCO BRL, Gaithersburg, MD). A 1,338-bp RACE product was obtained and cloned (pCMB314). The nucleotide sequence was determined and revealed to contain, except for the last 30 bp at the 3′ end, the full sequence contained in pCMB14 and an additional N-terminal portion that rendered a complete ORF of 1,173 bp (Fig. 2). A comparison of the deduced amino acid sequence with those found in databases, done with the BLASTX algorithm (Gish and States 1993; Altschul et al. 1990), showed that the ORF contained in pCMB314 had a 55% identity to the predicted alfalfa ENOD8 gene product (Dickstein et al. 1993) and was nearly identical to the EP4 glycoprotein recently described by van Engelen et al. (1995). In keeping with these results, we designated the ORF contained in pCMB314 as inducible EP4 (iEP4) in order to distinguish this sequence from EP4, although it is highly probable that this gene is the same as the one described previously. When compared with the EP4 amino acid sequence, iEP4 had 3 changed amino acids; 2 of this changes are functionally conserved (L for W, L for I, and T for A at positions 233, 243, and 334, respectively). It can be observed that pCMB314 contained the full-length published EP4 sequence followed by a noncoding region of 108 bp at the 3′ end and 8 additional amino acid residues at the 5′ end. The proposed initiation ATG codon is flanked by a conserved consensus sequence proposed by Lutcke (1987) as the optimal for initiation in plants. This ATG start codon is preceded by a 54-bp untranslated sequence. The ORF codes for a 391 amino acid acidic protein (estimated pI [isoelectric point]: 4.66) with an estimated molecular mass of 42,638 Da (Fig. 2). Five potential N-glycosylation sites (Asn-X-Ser/Thr) were found at positions 63, 98, 146, 186, and 329 of the deduced amino acid sequence. Hydrophilicity analysis revealed a hydrophobic domain at the N-terminal portion of the predicted protein. According to Von Heijne (1983), the proteolytic cleavage site is located between the alanine and serine at position 23.

To obtain the genomic sequence of iEP4, a PCR amplification of genomic carrot DNA was carried out with primers DCC21 and DCC11 (5′-ATCAAGTAGTATCAAATACCATC-3′). A product of 2,180 bp was obtained and cloned in vector pGEM-T vector (Promega). Recombinant plasmid pCMB414 was obtained and sequenced. This genomic clone contained the entire cDNA sequence cloned in pCMB314 plus four intervening sequences of 72, 496, 111, and 168 bases, respectively, indicated by lowercase letters in Figure 2. In all four introns the conserved sequences at the splicing junctions, exon/GT-intron-AG/exon, characteristic of eucaryotic pre-mRNA (Jacob and Gallinaro 1989), are present.

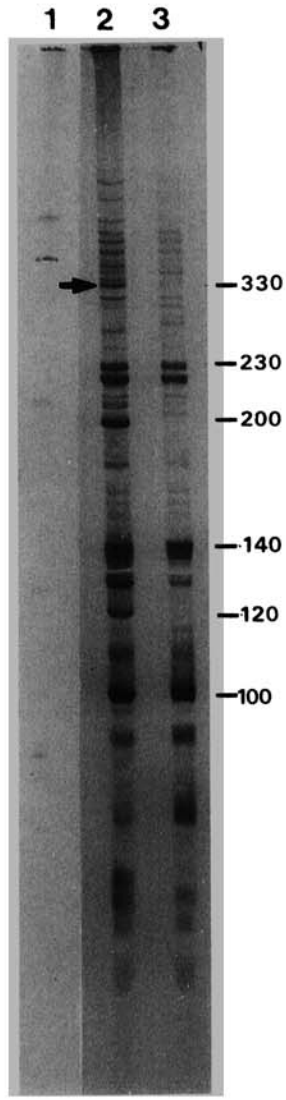

Fig. 1. Radioautography of differential display reaction products obtained with total RNA from carrot cells. Total RNA was extracted from carrot cells after 7 h of exposure to a heat-released elicitor (HRE). Reverse-transcriptase reaction was carried out with $T_{11}GC$ as primer. Polymerase chain reaction (PCR) reactions were carried out on single-strand cDNA with $T_{11}GC$ and oligo 5. Lane 1, control PCR reaction without cDNA; lane 2, RNA from cells treated with HRE; lane 3, RNA from nontreated control cells. Arrow indicates 300-bp product.

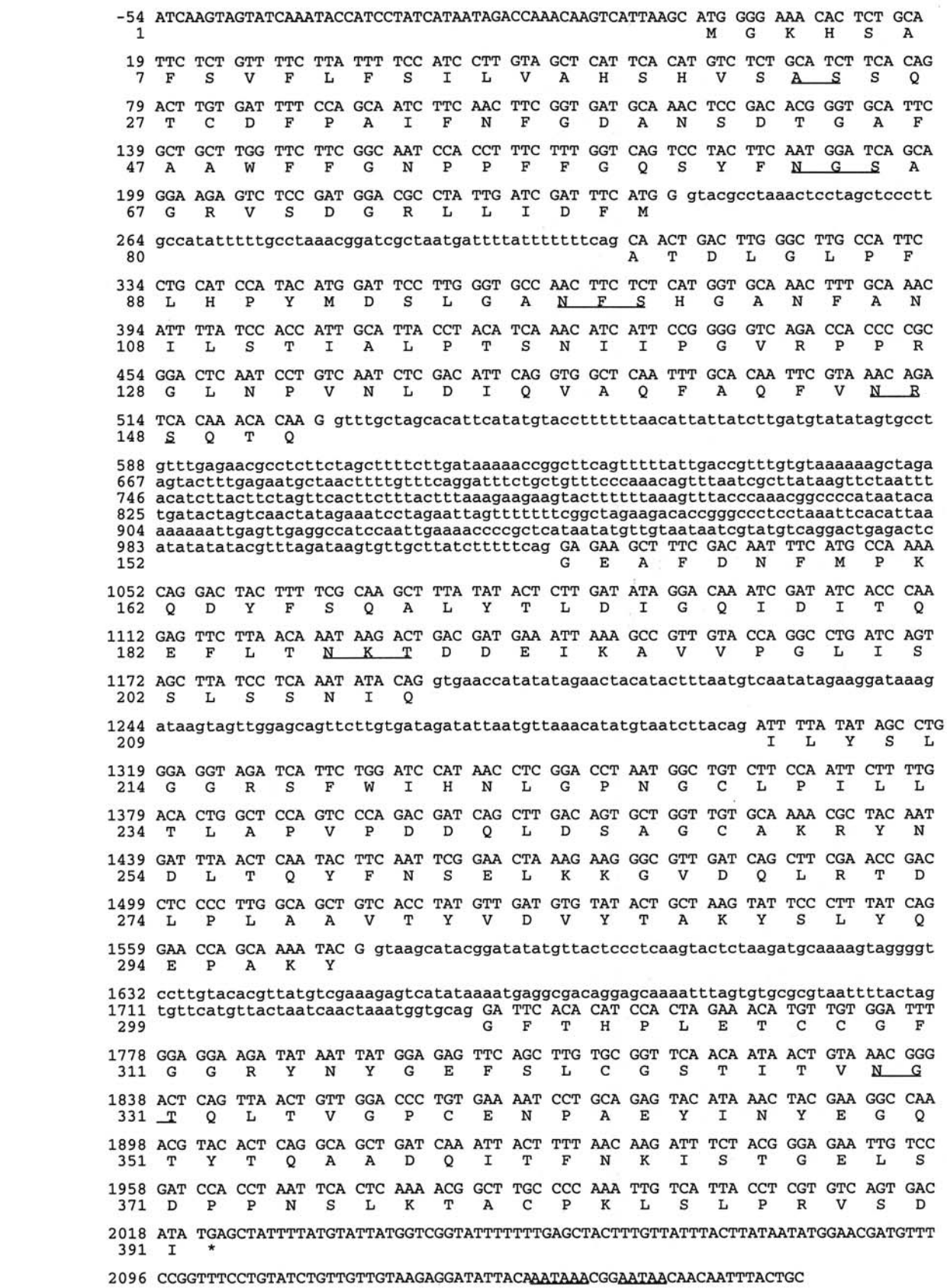

Fig. 2. Genomic nucleotide sequence and deduced amino acid sequence of iEP4. Nucleotides are numbered from the initiation codon. Introns are shown in lower-case letters. Potential N-glycosylation sites and poly-adenylation signals are underlined. The TGA stop codon is indicated by an asterisk. The original 308-bp pCMB14 clone is between nucleotides 1853 and 2161. The potential cleavage site between amino acids 23 and 24 is underlined.

To confirm that treatment with the fungal elicitor truly increases the level of expression of iEP4 mRNA, a Northern (RNA) blot analysis was carried out. Ten micrograms of total RNA used for the differential display reaction was analyzed on an RNA gel blot with the complete iEP4 cDNA clone contained in plasmid pCMB314 as a probe (Fig. 3B). According to ethidium bromide and methylene-blue staining of rRNAs on the gel and blot, respectively (Fig. 3A), the amount of total RNA was the same in both samples. An RNA of approximately 1,300 bp present on HRE-treated cells hybridized with the probe (Fig. 3B, lane 2), and no signal could be seen on untreated control cells (Fig. 3B, lane 1). The same RNA gel blot was washed and subsequently hybridized with a carrot actin fragment as a probe in order to test the effect of HRE on a housekeeping gene (Fig. 3C). A double hybridization signal that migrated in front of the ribosomal small subunit with equal intensity in HRE-treated and control cells was observed.

Similar results were obtained with a reverse-transcription PCR method (RT-PCR) to monitor gene expression. Primers DCC10 (5′-GAAAATCCTGCAGAGTACATAAAC-3′) and DCC20 (5′-CCGTTTATTTGTAATATCCTCTTA-3′) were designed to amplify a 280-bp fragment of iEP4 mRNA. As shown in Figure 4A, when two different amounts of cDNA were used to carry out the PCR, the expected 280-bp fragment was observed only with cDNA prepared from RNA of HRE-treated cells. (Fig. 4A, lanes 3 and 5). PCR amplification of the same cDNA with primers ACT 1 (5′-TCTGCTGGAATCC ATGAAACTACTT-3′) and ACT 2 (5′-CGAGATCCACATCT

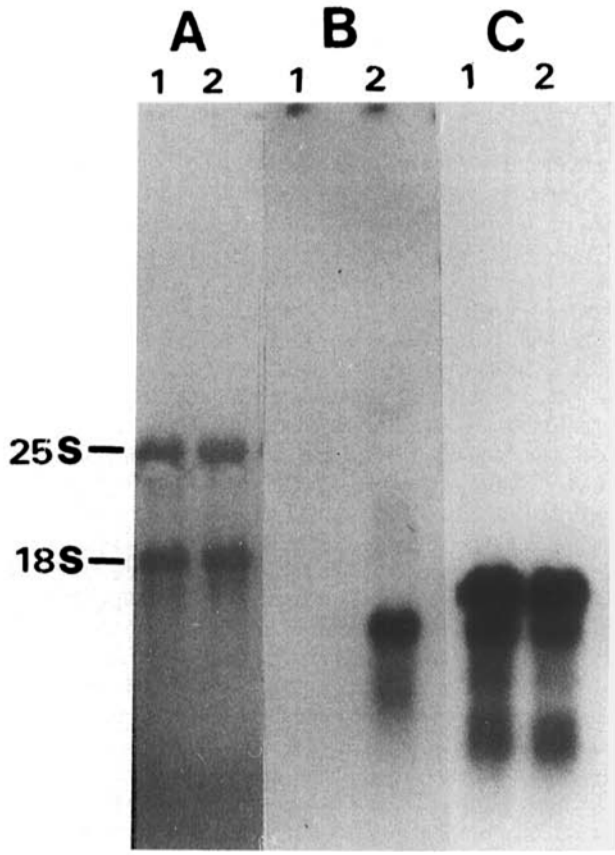

Fig. 3. Northern (RNA) blot analysis of iEP4 and actin mRNA expression in carrot cell cultures. Ten micrograms of total RNA isolated from control cells (lane 1) and from elicitor-treated cells (lane 2) was examined by RNA blot hybridization with an iEP4-specific probe (**B**) and an actin probe (**C**). **A,** Membrane stained with methylene-blue before being hybridized.

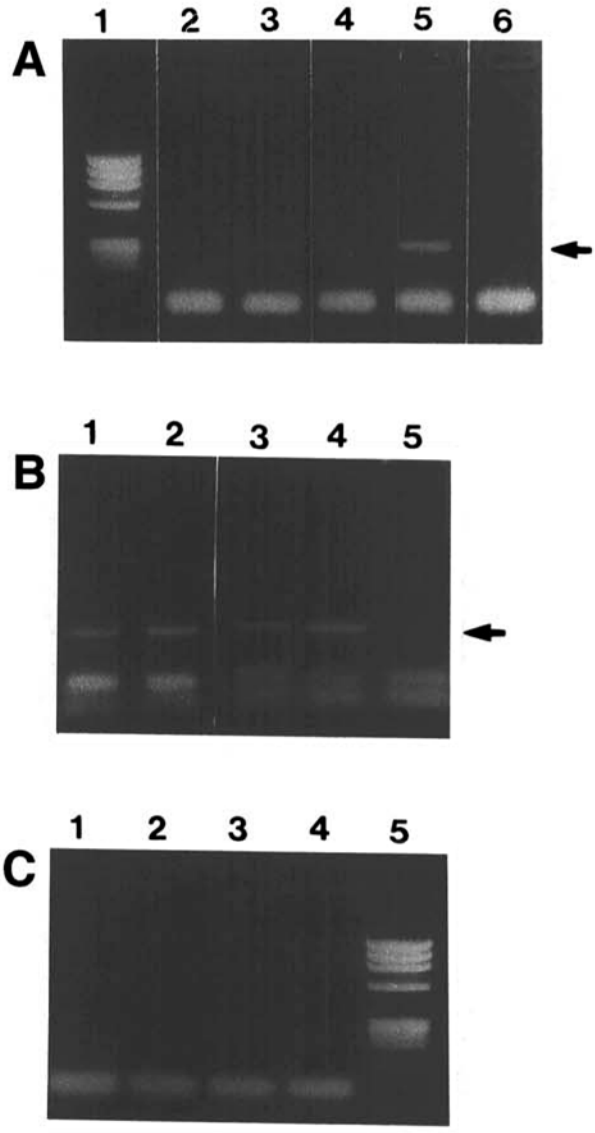

Fig. 4. Analysis of gene expression by reverse-transcriptase polymerase chain reactin (RT-PCR). Reverse-transcriptase reaction was carried out on total RNA obtained from heat-released elicitor (HRE)–treated and water-treated (control) cells with the $T_{11}GC$ primer. Aliquots of cDNA were submitted to PCR with specific primers for iEP4, actin, and calmodulin genes. PCR products were analyzed on 1.6% agarose gels and stained with ethidium bromide. Arrows indicate position of expected fragments. **A,** Expression of iEP4. 0.01 µl of cDNA samples (lanes 2 and 3) and 0.02 µl (lanes 4, 5, and 6) from HRE-treated cells (lanes 3 and 5) or control cells (lanes 2 and 4) were submitted to PCR with primers DCC10 and DCC20. Lane 1, ØX174/*Hae*III size marker. Lane 6, control reaction with no cDNA. **B,** Expression of actin. cDNA samples of 0.01 µl (lanes 1 and 2) and 0.02 µl (lanes 3 and 4) from HRE-treated cells (lanes 2 and 4) or control cells (lanes 1 and 3) were submitted to PCR with ACT1 and ACT2 as primers. Lane 5, control reaction with no cDNA. **C,** Expression of calmodulin. cDNA samples of 0.01 µl (lanes 1 and 2) and 0.02 µl (lanes 3 and 4) from HRE-treated cells (lanes 2 and 4) or control cells (lanes 1 and 3) were submitted to PCR with CAL1 and CAL2 as primers. Lane 5, ØX174/*Hae*III size marker. Arrows on right indicate position of expected fragments.

GCTGGAAGGTA-3′), corresponding to the housekeeping actin gene, is shown in Figure 4B. It can be observed that with both concentrations of cDNA the expected 270-bp actin fragment (arrow) was amplified with RNA from control and HRE-treated cells (Fig. 4B, lanes 1 to 4), thus indicating that, under these experimental conditions, as observed in the Northern blot analysis of Figure 3, iEP4 mRNA was detected only in HRE-treated cells. No cDNA corresponding to calmodulin mRNA was amplified with primers Cal 1 (5′-GCGGCCGCC TCAAATCATATCATA-3′) and Cal 2 (5′-TGGTGATGGGC AGATCAACTATGA-3′) with RNA from control or HRE-treated cells (Fig. 4C). This is an expected result since no GC residues immediately upstream of the poly (A) tail were described in this gene (GenBank accession number DCCAM1G) and consequently no cDNA should be synthesized during reverse transcription with primer 5′-T_{11}GC-3′. This result revealed that no DNA contamination was present in the amplified samples and that only a subset of mRNAs was reverse transcribed and consequently detected by this method.

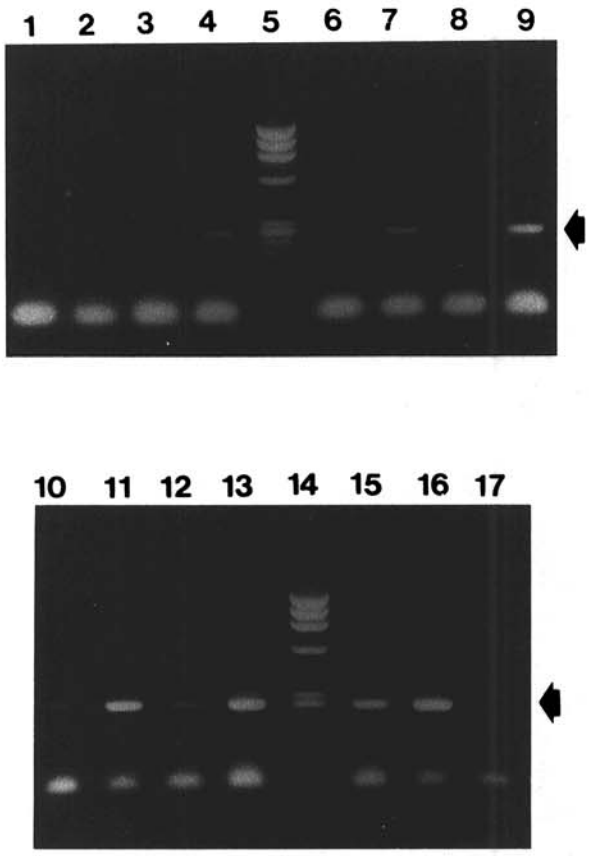

Fig. 5. Analysis of the level of expression of iEP4 mRNA in control and heat-released elicitor (HRE)–treated carrot cells. Total RNA (5 μg) obtained from control and HRE-treated cells was subjected to reverse transcription with primer T_{11}GC. Different amounts of the reverse transcriptase reaction were submitted to polymerase chain reaction (PCR) with the primers DCC10 and DCC20 to amplify a 280-bp fragment of iEP4 mRNA. PCR products were subjected to 1.6% agarose gel electrophoresis and stained with ethidium bromide. Lanes 1 (0.01 μl), 3 (0.02 μl), 6 (0.05 μl),8 (0.1 μl), 10 (0.2 μl), 12 (0.5 μl), and 15 (1 μl) correspond to control cells. Lanes 2 (0.01 μl), 4 (0.02 μl), 7 (0.05 μl), 9 (0.1 μl), 11 (0.2 μl), 13 (0.5 μl), and 16 (1 μl) correspond to HRE-treated cells. Lanes 5 and 14 are the *Hae*III digested Øx174 size markers. Lane 17, PCR reaction was carried out without cDNA. Arrows on right indicate position of expected fragments.

Recently, van Engelen et al. (1995) reported that normal carrot cells secreted the glycoprotein EP4a into the culture medium. Compared with this report, our result—that iEP4 mRNA was only present in HRE-treated cells—was unexpected. In order to study this apparent contradiction, the experiment shown in Figure 5 was carried out. A reverse-transcription reaction was carried out with the primer 5′-T_{11}GC-3′ on total RNA from control and HRE-treated cells. Samples of the reverse-transcription reaction (cDNA) were submitted to PCR with the DCC10 and DCC20 primers to amplify a 280-bp fragment of iEP4 mRNA. As shown in Figure 5, when PCR reactions were carried out with samples of 0.01 to 0.1 μl of the reverse-transcription reaction mixture (Fig. 5, lanes 1 to 9) the 280-bp product (arrow) was only observed with RNA from HRE-treated cells; with 0.2-μl samples (Fig. 5, lanes 10 and 11) a small amount of the 280-bp product was formed with RNA from control and HRE-treated cells; with 1-μl samples, a strong and almost identical signal was obtained with both samples (Fig. 5, lanes 15 and 16). These results showed that iEP4 mRNA was present in noninduced cells at very low concentration and that the presence of HRE increased the level of expression five- to 10-fold. These results are in agreement with those reported by van Engelen et al. (1995), who detected EP4a glycoprotein in the supernatant of carrot cell cultures. These results show that RT-PCR is a powerful tool to study the level of expression of low copy number eucaryotic mRNA. When compared with the expression of the actin gene, the basal expression level of iEP4 was very low, and was undetectable in Northern blot experiments. Therefore, RT-PCR should be the screening method of choice to study genes with low expression levels. This procedure allows us to screen a large number of induced mRNAs relatively quickly and at reasonably low cost, and it is far simpler than subtractive or differential hybridization to identify unknown differentially expressed genes (Bauer et al. 1993).

The high homology of iEP4 to the early alfalfa nodulin ENOD8 (Dickstein et al. 1993) is interesting, since it is thought that some of these early nodulins might indicate an early plant response to *Rhizobium.* On the other hand, the extracellular matrix of plant cells is the first line of defense against pathogens (Bowles 1990). A number of proteins are known to accumulate in response to wounding and infection, including glycine rich proteins (GRPs), peroxidases (Båga et al. 1995), cinnamyl alcohol dehydrogenase (Walter et al. 1988), callose synthetase (Kauss 1987), and hydroxyproline-rich glycoproteins (HRGPs) such as carrot extensin (Chen and Varner 1985). In 1992, Satoh et al. showed that suspension-cultured cells of carrot synthesize and secrete EDGP, an extracellular dermal protein that is induced in carrot tap root by wounding. Nevertheless, the accumulation of EDGP mRNA was not enhanced by the infection of carrots with the pathogen *Erwinia carotovora.*

As occurs with EDGP, EP4a glycoprotein is also secreted into the medium, but we were able to show that its level of transcription is greatly increased when the cells are exposed to a pathogen elicitor. This is in agreement with the generally accepted view that the cell wall and its associated proteins might be important in plant development and defense. Van Engelen et al. (1995) suggested that this glycoprotein might be an enzyme, although its putative enzymatic activity has not

been found so far. The biological activities of Nod factors and their structural similarity to fungal chitosan elicitors have prompted speculations that these may in fact be elicitors of the symbiotic response, and that the recognition- and signal-transduction processes leading to pathogenic and symbiotic plant responses may be similar. Indeed, Nod factor–induced developmental changes in the early phase of the symbiotic interaction share features of a plant defense response (Baron and Zambryski 1995). It is tempting to speculate, due to the high homology shared by iEP4 with ENOD8, that this glycoprotein might be a bacteria (*Rhizobium*) and/or fungal (*S. sclerotiorum*) plant response protein.

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