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Article *in* Plant Molecular Biology · January 1997

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Structure of the parsley caffeoyl-CoA *O*-methyltransferase gene, harbouring a novel elicitor responsive *cis*-acting element

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Received 2 February 1996; accepted in revised form 14 October 1996

Key words: Petroselinum crispum L., plant stress response, *O*-methyltransferase, gene regulation, footprints, *cis*-regulatory elements

Abstract

The sequence of the S-adenosyl-L-methionine:*trans*-caffeoyl-CoA *O*-methyltransferase (CCoAOMT, EC 2.1.1.104) gene, including the 5′-flanking region of 5 kb, was determined from parsley (*Petroselinum crispum*) plants. The enzyme appears to be encoded by one or two genes, and the ORF is arranged in five exons spaced by introns from 107 to 263 bp in length. The genomic sequence matches the ORF of the cDNA previously reported from elicited parsley cell cultures, showing only three base changes that do not affect the enzyme polypeptide sequence. S1 nuclease protection assays and primer extension analyses with genomic and cDNA templates revealed the transcription start site 67 bp upstream of the translation start codon, indicating a shorter 5′-UTR than reported previously for the transcript. Promoter regulatory consensus elements such as two 'CAAT' boxes and one 'TATA' box were identified at –196, –127 and –31, respectively, relative to the transcription start site, and an SV 40-like enhancer element is located 347 bp upstream. Most notably, three putative *cis*-regulatory elements were recognized by sequence alignments, which represent motifs recurring in the promoters of several genes of the stress-inducible phenylpropanoid pathway (boxes P, A and L). Transient expression assays with a set of 5′-truncated promoter-GUS fusions show that significant promoter activity is retained in a 354 bp promoter fragment. *In vitro* DNase l footprint experiments and electrophoretic mobilty shift assays (EMSA) identified in this fragment a unique sequence motif with elicitor-inducible *trans*-factor binding activity, which was unrelated to boxes P, A, or L. This novel *cis*-regulatory element, designated box E, appears to be conserved in the TATA-proximal regions of other stress-inducible phenylpropanoid genes, and *in vitro* binding of nuclear protein was confirmed in EMSA assays for such an element from the PAL-1 promoter (–54 to –45). Moreover, the deletion of box E reduced the activity and erased the elicitor-responsiveness of the CCoAOMT promoter in transient expression assays. The results corroborate the proposed physiological function of CCoAOMT in elicited plant cells and may shed new light on the sequential action of *trans*-active factors in the regulation of phenylpropanoid genes.

Introduction

Many plants respond to pathogen infection or environmental stress conditions by the induction of their phenylpropanoid metabolism. This activation provides the precursors for the production of furanocoumarin [53], pterocarpan [8] or stilbene phytoalexins [26]. However, many plants also utilize their phenylpropanoid metabolism by inducing the reinforcement and lignification of the cell wall [36, 40]. The early stage of this process is characterized by the esterification of cell wall polysaccharides with hydroxycinnamic acids, which commences almost instantaneously upon fungal challenge [22, 36] and renders the cells inaccessible to enzymatic lysis. Subsequent crosslinking

The nucleotide sequence data reported will appear in the GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number Z54183 (PCCCOAMTR).

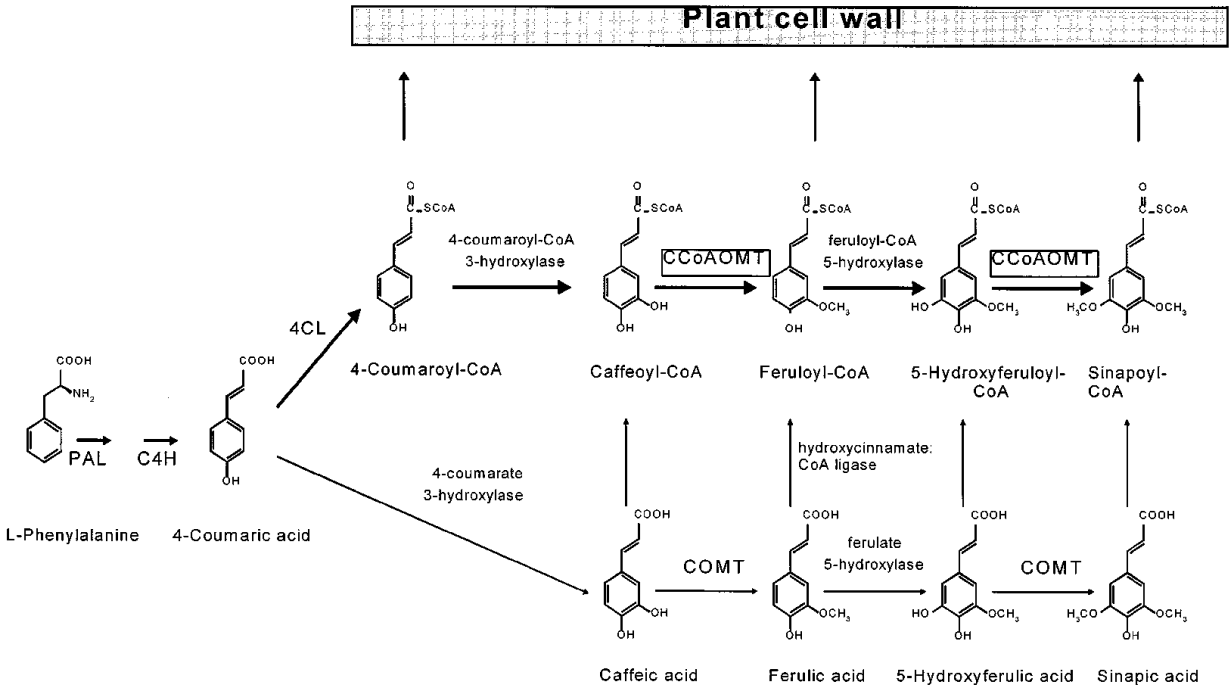

Figure 1. Biosynthesis of hydroxycinnamoyl-CoAs. Feruloyl- and sinapoyl-CoA are formed in parsley cells by CoA-ligation of 4-coumaric acid followed by methoxylations at the CoA-ester stage (upper path). *S*-adenosyl-L-methionine:caffeoyl-CoA 3-*O*-methyl transferase (CCoAOMT) catalyses both methylations in this pathway, which serves in lignification as well as stress compensation. The formation of hydroxycinnamoyl-CoAs had previously been considered to derive exclusively from CoA ligation of the corresponding hydroxycinnamic acids (lower path).

of the hydroxycinnamoyl moieties protects the cells further against mechanical damage and pathogen invasion. Furthermore, the cell wall esters presumably fulfill other functions in stress compensation such as oxygen radical scavenging, release of hydroxybenzaldehydes as potential 'phytoalexins of the cell wall' [36] and they may provide the substrates or anchoring sites for lignification [27, 36]. In several instances, the genetics of efficient cell wall reinforcement has been correlated with the resistance character of plant cultivars [1, 45, 52]. Obviously, the mechanisms involved have considerable impact on the agrochemical industry that aims at exploiting signal transduction pathways for crop plant protection.

During the disease resistance response, plants incorporate 4-coumaroyl and feruloyl esters into their cell wall [36, 40], and the feruloyl moiety is a major component of lignin in higher plants [27]. Thus, both cell wall reinforcement and lignification specifically require the enhanced synthesis of feruloyl-CoA, and it is likely that lignification of the cell wall follows esterification in the disease resistance response of plants [36, 40]. The formation of feruloyl-CoA was previously assumed to depend on the methylation of caffeic acid and subsequent ligation of ferulic acid to CoA. However, we have identified a novel biosynthetic route which proceeds via hydroxylation and subsequent methylation of 4-coumaroyl-CoA (Fig. 1). The caffeoyl-CoA *O*-methyltransferase involved (CCoAOMT) has been characterized in detail and the cDNA was cloned [42, 43, 49]. The CoA-ester-dependent pathway initially studied in parsley has meanwhile been confirmed for additional plants such as safflower, carnation, grapevine (unpublished results), *Zinnia* [55] and *Arabidopsis* [58]. It is likely to be distributed ubiquitously in higher plants. Moreover, the homologous CCoAOMT transcripts are specifically induced in the vascular tissues of parsley and *Zinnia* plants as well as in *Zinnia* cell cultures during lignification [55]. The formation of sinapoyl-CoA by methylation of 5-hydroxyferuloyl-CoA has been proven in parallel studies (Kneusel and Matern, unpublished results). Thus, the sequential methoxylations of 4-coumaroyl-CoA bridge the gap between the general phenylpropanoid pathway and the specific lignin path-

way that requires the reduction of the CoA esters to the corresponding monolignols [27].

Most of the relevant molecular investigations have focused on the regulation of enzymes of the general phenylpropanoid pathway, i.e. phenylalanine ammonia-lyase (PAL) and 4-coumarate: CoA ligase (4CL) [4, 32, 33, 41]. These enzymes were shown to be induced *de novo* in the disease resistance response of plants as well as by irradiation with UV-light [12, 25] or several other kinds of stress conditions [9, 25, 32, 41]. Many investigations were initially conducted in systems of reduced complexity, employing cell cultures instead of differentiated plants and similarly fungal elicitor fractions rather than live fungi. The picture emerging from these studies shows that conserved *cis*-regulatory elements are present in the promoters of both PAL and 4CL genes, and, on the basis of *in vitro* and *in vivo* footprint experiments, stress-responsive elements were distinguished from constitutive regulatory elements [13, 32, 41]. These studies have been extended to transgenic plants, and *trans*-acting factors binding to the promoter footprint regions are under investigation [5, 56]. PAL and 4CL catalyse reactions very early in the biosynthetic pathways that lead to different branches of phenylpropanoids and thus respond to multiple stimuli. Accordingly, the observed regulatory patterns are very complex and may involve, for example, the differential induction of isoenzymes in case of PAL [4] and the combinatorial interaction of several, spatially separated promoter elements including exonic sequences in case of 4CL [15, 28, 30].

Induction experiments and kinetic data [42, 49] had revealed that CCoAOMT is also a highly regulated enzyme, which, *in vivo*, might even control the flux of 4-coumarate into cell wall esters and lignin. The regulatory properties of the methyltransferase and the fact that it is more specifically involved in cell wall polymer formation than either PAL or 4CL caught our attention. We describe in this report the parsley CCoAOMT gene, including an extended 5′-flanking region, as well as putative *cis*-regulatory elements that classify the enzyme as a typical member of the inducible phenylpropanoid pathway. Moreover, a novel *cis*-regulatory element was pinpointed in the CCoAOMT promoter on the basis of *in vitro* DNA-protein interactions and transient expression assays, and the functional relevance of this element is corroborated by alignment studies.

Materials and methods

Enzymes, vectors and chemicals

Enzymes and phage and plasmid vectors were purchased from Boehringer Mannheim (Pensberg, FRG), Pharmacia (Freiburg, FRG), BRL (Karlsruhe, FRG) or Promega, Stratagene and U.S. Biochemicals (Serva, Heidelberg, FRG). Dynabead Oligo$(dT)_{25}$ were purchased from Dynal (Hamburg, FRG).

Plant materials and culture conditions

Plants of *Petroselinum crispum* were grown from seeds in the greenhouse (12 h day and night time conditions), the youngest leaves were harvested 3–4 months later for isolation of the genomic DNA. Parsley cell suspension cultures were propagated in the dark and treated 6 days after subculturing with 4 mg per 40 ml culture volume with crude cell wall elicitor from *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*) as described previously [49]. The cells were harvested 4 h after addition of the elicitor by vacuum filtration, frozen in liquid nitrogen and stored at –70 °C until use.

Isolation of RNA

Total RNA was isolated from dark-grown parsley cell cultures that had been treated for 4 h with *Pmg* elicitor. Poly$(A)^+$ mRNA was isolated from parsley cells by extraction with magnetic dT-conjugated microspheres (Dynabeads) [14].

Construction and screening of a genomic library

High-molecular-weight genomic DNA was isolated from parsley leaves by a procedure described elsewhere [7, 51]. DNA fragments of about 12 to 20 kb were generated by partial *Sau*3AI digestion and size fractionation through sucrose gradient centrifugation as described for maize DNA [50], and a genomic library was constructed from these fragments in λGEM11 (Promega), a derivative of λEMBL3 [11] and λ2001 [21]. Independent recombinant phages were plated on *Escherichia coli* KW251 at a density of 60 000 pfu per 230 mm × 230 mm plates, and the plaques were transferred to nylon Nytran filters (Schleicher & Schüll, Dassel, FRG) for hybridization [18]. Positive phages were selected from 0.8 million recombinants through three rounds of screening using standard methods [47]

with the labeled CCoAOMT cDNA pL2-4 as a probe [49].

Nucleotide sequencing

On the basis of restriction maps of the genomic clones, the CCoAOMT gene was encoded in a 4.7 kb *Ava*II fragment and a 5.0 kb *Sac*I/*Hin*dIII fragment. These fragments were subcloned into the plasmid vectors pUC18 [38] and pBluescript II (Stratagene). The *Sac*I/*Hin*dIII fragment was further restricted with *Eco*RI and the resulting fragments were individually subcloned. A series of nested deletions derived from the *Eco*RI/*Hin*dIII insert and a portion of the *Sac*I/*Hin*dIII fragment in pBluescript were obtained by the exonuclease III method of Henikoff [17]. In addition, a portion of the 4.7 kb *Ava*II fragment was sequenced using several synthetic oligonucleotide primers derived from known sequences. DNA sequencing was carried out by the dideoxy-chain termination method [48] using double-stranded plasmid DNA [24] and the USB sequenase sequencing kit (U.S. Biochemicals).

Southern hybridization

Genomic DNA (10 μg) isolated from parsley leaves was digested with either *Eco*RV, *Hin*dIII or a combination of *Eco*RI with *Eco*RV; the restriction fragments were separated by electrophoresis in an 0.7% agarose gel and blotted onto nitrocellulose membrane by downward capillary transfer [57]. The DNA was fixed to the membrane by UV irradiation (Stratalinker, Stratagene), and the filters were prehybridized for 4 h at 68 °C in 6× SSC, 5× Denhardt's solution, 0.5% SDS and 0.1 mg/ml herring sperm DNA [47]. The filters were subsequently hybridized with the labelled *Hin*dIII insert (2.6 kb) spanning the entire CCoAOMT genomic sequence. Hybridization was continued overnight at 68 °C, and the filters were washed three times at room temperature with washing buffer (2× SSC, 0.1% SDS) for five min each followed by two 30 min washes at 68 °C in the same buffer, prior to autoradiography.

Primer extension

Poly(A)$^+$ mRNA (2 μg) isolated from elicited parsley cells was used as a template for reverse transcription with oligonucleotide primer NM1 (Fig. 4). The mRNA was denatured for 2 min at 95 °C prior to annealing by incubation at 60 °C for 10 min and cooling down to <35 °C over 10 min with 0.8 pmol oligonucleotide NM1 in 15 μl reaction mix (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP). After annealing, 10 units ribonuclease inhibitor and 100 units MMLV reverse transcriptase were added and the mixture was incubated for 40 min at 37 °C. Unincorporated primer was removed with the Qiaex PCR purification kit (Qiagen, Hilden, FRG). The primed transcripts were precipitated with ethanol and the pellet was resuspended in formamide buffer (95% formamide, 20 mM EDTA, 0.05% xylene xylanol FF and 0.05% bromophenol blue) and applied to a 6% polyacrylamide sequencing gel for size determination. A sequencing reaction with primer NM1 and the *Ava*II fragment (Fig. 2) was used as molecular weight marker.

S1 nuclease mapping

Both a genomic fragment and a fragment derived from the previously cloned cDNA [49] were used as probes. The 213 bp genomic fragment was obtained by digestion of the 4.7 kb *Ava*II insert with *Dpn*I and *Hin*fI. The *Hin*fI site is located in the first exon of the CCoAOMT gene 16 bp downstream of the translation start site. The 521 bp cDNA fragment was generated from the insert pL2-4 [49] by digestion with *Nco*I, thus stretching from the left border *Eco*RI site to 150 bp downstream of the translation start site. The probes were 5′-labelled with [(γ-^{32}P]ATP at the *Hin*fI and *Nco*I site, respectively. Poly(A)$^+$ mRNA (1 μg) and 4 × 10^5 cpm of either one of the probes were denatured at 85 °C and annealed at 42 °C for 12 h in 20 μl hybridization buffer [47]. S1 nuclease digestion was accomplished at 20 °C for 30 min by the addition of 80 units S1 nuclease in 200 μl S1-buffer [47], the DNA/RNA hybrids were extracted with phenol-chloroform and precipitated subsequently with ethanol, and the pellet was resuspended in formamide buffer for size determination on a 6% sequencing gel. A sequencing reaction with pBluescript (Stratagene) as template and the reverse primer (5′-CAGGAAACAGCTATGAC-3′) was employed as molecular weight marker.

Preparation and transformation of protoplasts

Protoplasts were prepared from 5-day old parsley cell cultures as described by Dangl *et al.* [6], and 6 × 10^6 protoplasts were transformed with 100 μg of circular plasmid DNA by electroporation as described by Renelt *et al.* [46]. Slightly modified parameters were

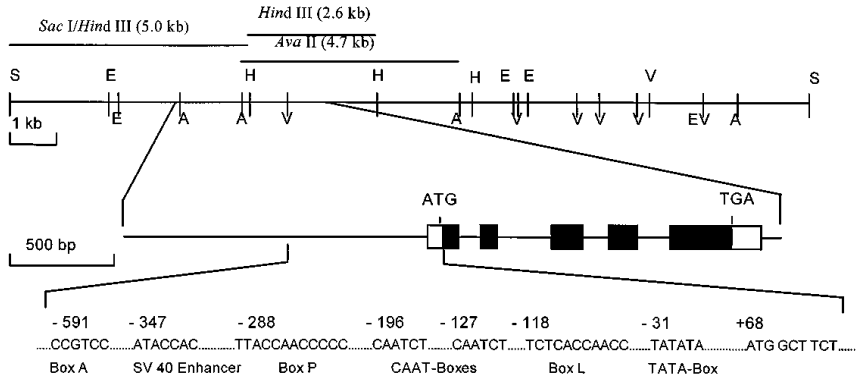

Figure 2. Structural organization of the CCoAOMT gene from parsley. The restriction map (A, *Ava*II; E, *Eco*RI; V, *Eco*RV; H, *Hin*dIII; S, *Sac*I) is outlined schematically, and the prominent regulatory elements are indicated at their upstream position relative to the transcription start site. Filled and open boxes represent the coding region and the 5′- and 3′- untranslated regions, respectively.

employed (325 V/cm, 125 μF instead of 750 V/cm and 25 μF), which enhance the transformation efficiency (H. Frohnmeyer, Freiburg, personal communication). Following transformation, the protoplasts were treated with crude *Pmg* elicitor (100 μg/ml protoplasts) and incubated at 26 °C in darkness for 10 h in 6 cm Petri dishes. GUS activity was determined using a fluorometric assay with 4-methylumbelliferylglucuronide [19].

Deletion of promoter elements

Deletions of box L or E were accomplished by recombinant polymerase chain reactions [44] with chimeric primers, which pair with both sides flanking the deleted sequences. The chimeric primer for deletion of box L was 5′-GTGGGACCCATCAAGATC**T**CAGACCGG-GTTGGTG-3′, which induced the replacement of a 20 bp-sequence, representing box L, in the CCoAOMT promoter by a single T and thus created a *Bgl*II restriction site for selection of mutant constructs. The chimeric primer for deletion of box E was 5′-CCAAGTTTATACCAAACG**AG**ATCTCCAT-CTCACCAAC-3′. Likewise, this primer replaced an 18 bp-deletion, representing box E, by 5′-AG-3′ and created a *Bgl*II restriction site. The underlining of nucleotides symbolizes sequence identity with the CCoAOMT promoter.

Preparation of nuclear extracts

Nuclear extracts were prepared from dark-grown parsley cells by a modification of a method used for isolation of tomato nuclei [35]. The frozen cells (15 g) were ground to a fine powder in liquid nitrogen, suspended in 3 volumes (v/w) of homogenization buffer (250 mM sucrose, 10 mM NaCl, 25 mM Pipes pH 7.0, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 20 mM 2-mercaptoethanol, 0.1% Nonidet P-40, and 0.2 mM phenylmethylsulfonyl fluoride) and stirred in a glass beaker until the cell powder was completely dissolved in the buffer. The homogenates were filtered through two layers of nylon mesh (100 μm pore size) and subsequently through one layer of 20 μm. The filtrates were centrifuged for 20 min at 4200 $\times$ *g* at 4 °C. The nuclei were washed four times by suspending the pellets in 1.5 vol. (v/w) of homogenization buffer followed by centrifugations at 1900 $\times$ *g* for 10 min, and 1400 $\times$*g* for 10, 8, and 6 min. Lysis and protein extraction were performed as described elsewhere [35].

Site-specific labelling of DNA fragments

For EMSAs and DNase I footprinting analysis the promoter fragment of parsley CCoAOMT from -354 to +40 (*Hin*dIII), was restricted with *Bsp* 119I (–55), blunted with Klenow, and the resulting fragment (–354/–52) was cloned into the *Sma*I site of pBluescript. Site specific labelling of the appropriately orientated P/L probe with [α-^{32}P]dATP was performed after digestion with *Not*I and *Eco*RI at the latter restriction site to a specific activity of ca. 5 $\times$ 10^7 dpm/μg DNA. The resulting fragment from –354 to –52, still containing the boxes P and L, was designated as P/L probe. The double-stranded oligonucleotide probes (box E and PAL1 E) with engineered restriction site overhangs were prepared by annealing complementary single-stranded oligonucleotides and

were labelled with [α-^{32}P]dATP to a specific activity of ca. 2×10^8 dpm/μg DNA.

Gel retardation experiments

The standard electrophoretic mobility shift assay (EMSA) was performed in a volume of 15 μl using ^{32}P-labelled DNA fragments (1.5 fmol P/L probe or 3 to 6 fmol of the individual oligonucleotide probes, each 20 000 dpm). Binding was carried out in 1$\times$ binding buffer (20 mM Hepes, pH 7.5, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol) with 4 μg poly(dI-dC) $\cdot$ (dI-dC) 1 μg nuclear protein and, in some cases, different amounts of unlabelled competitor DNA. The binding mixture was incubated for 30 min at room temperature and loaded without the addition of dyes onto a 5% native polyacrylamide gel (19:1, acrylamide/bisacrylamide) prepared with electrophoresis buffer (25 mM Tris, pH 8.7, 190 mM glycine, 1.5 mM EDTA) and 2.5% glycerol. The gels were run at 4 °C in a cooling device at 10–15 V/cm, dried after electrophoresis and subjected to autoradiography.

DNase I footprinting reaction

The conditions for the DNase I footprinting were the same as those for the EMSAs except that the footprinting reactions contained 2 to 4 μg nuclear protein, 10 μg poly(dI-dC) $\cdot$ (dI-dC) and 3 fmol ^{32}P-labelled P/L probe in a total volume of 25 μl. After 30 min incubation at room temperature the mixture was brought to 3 mM Mg^{2+} with $MgCl_2$, followed by the addition of 1 unit DNase I (Stratagene) which was allowed to react for 1 min. The reactions were stopped by the addition of 100 μl stop solution (0.5 M NaCl, 10 mM EDTA, 0.1% SDS, and 50 μg/ml yeast RNA) and extracted once each with phenol-chloroform and chloroform. Nucleic acids were precipitated with ethanol, washed with 70% ethanol, resuspended in formamide loading dye and analyzed on a 6% denaturing polyacrylamide-urea sequencing gel after denaturing for 2 min at 95 °C. The ^{32}P-labelled P/L probe was subjected to G- and A>C-specific Maxam-Gilbert sequencing reactions [37] to obtain a sequencing ladder as reference.

Results

Isolation of the gene

A genomic library was established in λGEM11 using size-fractionated DNA digests from young parsley leaves, and six CCoAOMT clones were selected with the pl2-4 cDNA [49] probe from a total of 0.8 million recombinant clones. One of these clones was mapped and characterized further. The *Sac*I insert of ca. 17 kb was restricted with *Ava*II, and the resulting fragment of roughly 4.7 kb (Fig. 2), containing the complete CCoAOMT coding sequence, including 208 bp upstream of the translation start site, was subcloned in pUC18. For DNA sequencing, a 2.6 kb *Hin*dIII fragment was derived from this insert (Fig. 2) and subcloned independently. A 5 kb 5′-region of the λGEM11 *Sac*I insert, which included the complete promoter region, was cloned separately in pUC18 after restriction with *Hin*dIII (Fig. 2). This region was entirely sequenced and revealed together with that of the 2.6 kb insert of the *Ava*II/*Hin*dIII clone, the sequence of the CCoAOMT gene (Figs. 2 and 3).

Coding sequence and transcription start site

The sequence, part of which is shown in Fig. 3 (the full sequence is accessible in GenBank, accession number Z54183), revealed the exon-intron organization of the coding region. Five exons, separated by introns of 107, 263, 126 and 159 bp length, respectively, and the flanking regions of 216 bp downstream and 5 kb upstream were analyzed. The exon sequences matched the coding region of the cDNA previously identified from elicited parsley cultures [49] with only three base substitutions in positions +686 (C to T), +913 (C to T) and +962 (A to T) relative to the translation start site, all of which did not affect the previously reported enzyme polypeptide sequence. These marginal discrepancies may be attributed to the fact that different plant materials served for the isolation of the gene or cDNA. Matching sequences of the cDNA and the gene were also determined in the 3′-flanking region with two stop codons located at short distance (+1446 and +1558) and followed by the polyadenylation signal (AATATA) at position +1566 (Fig. 3). A major discrepancy, however, was observed between the 5′-UTR reported for the cDNA [49] and the genomic leader sequence since the homology ended 11 bp upstream of the translation start codon. Therefore, S1 nuclease protection assays were carried out using a

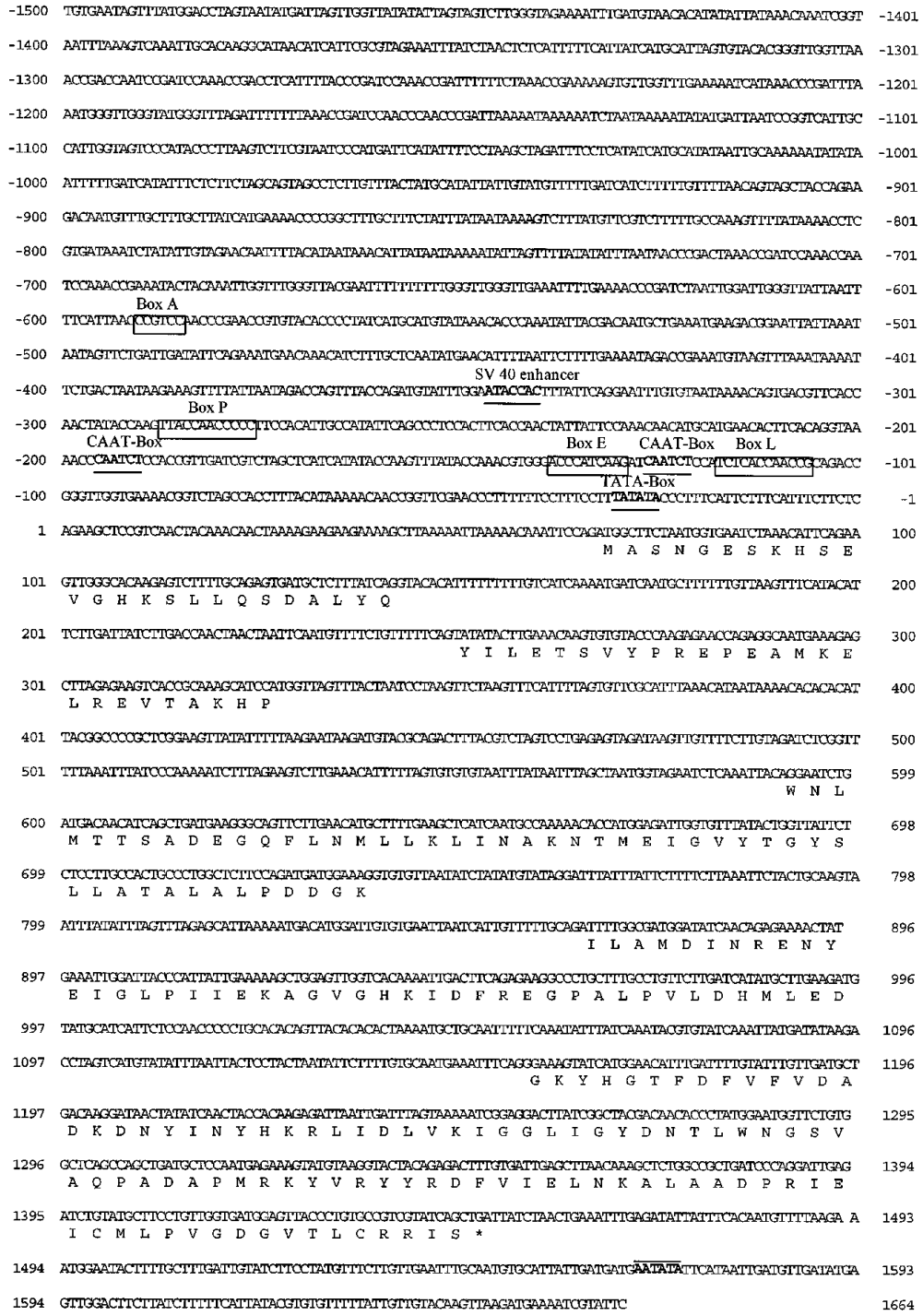

Figure 3. DNA sequence of the CCoAOMT gene with 3′- and 5′-flanking regions and deduced amino acid sequence. The transcription start site (+1) was assigned on the basis of S1 mapping and primer-extension analysis. Conserved eucaryotic promoter elements (TATA and CAAT boxes) and an inverted version of a SV 40 enhancer element are in bold type and underlined. Putative plant regulatory elements involved in UV light and/or elicitor regulation [31] are indicated by open boxes. The polyadenylation signal is marked by overlining.

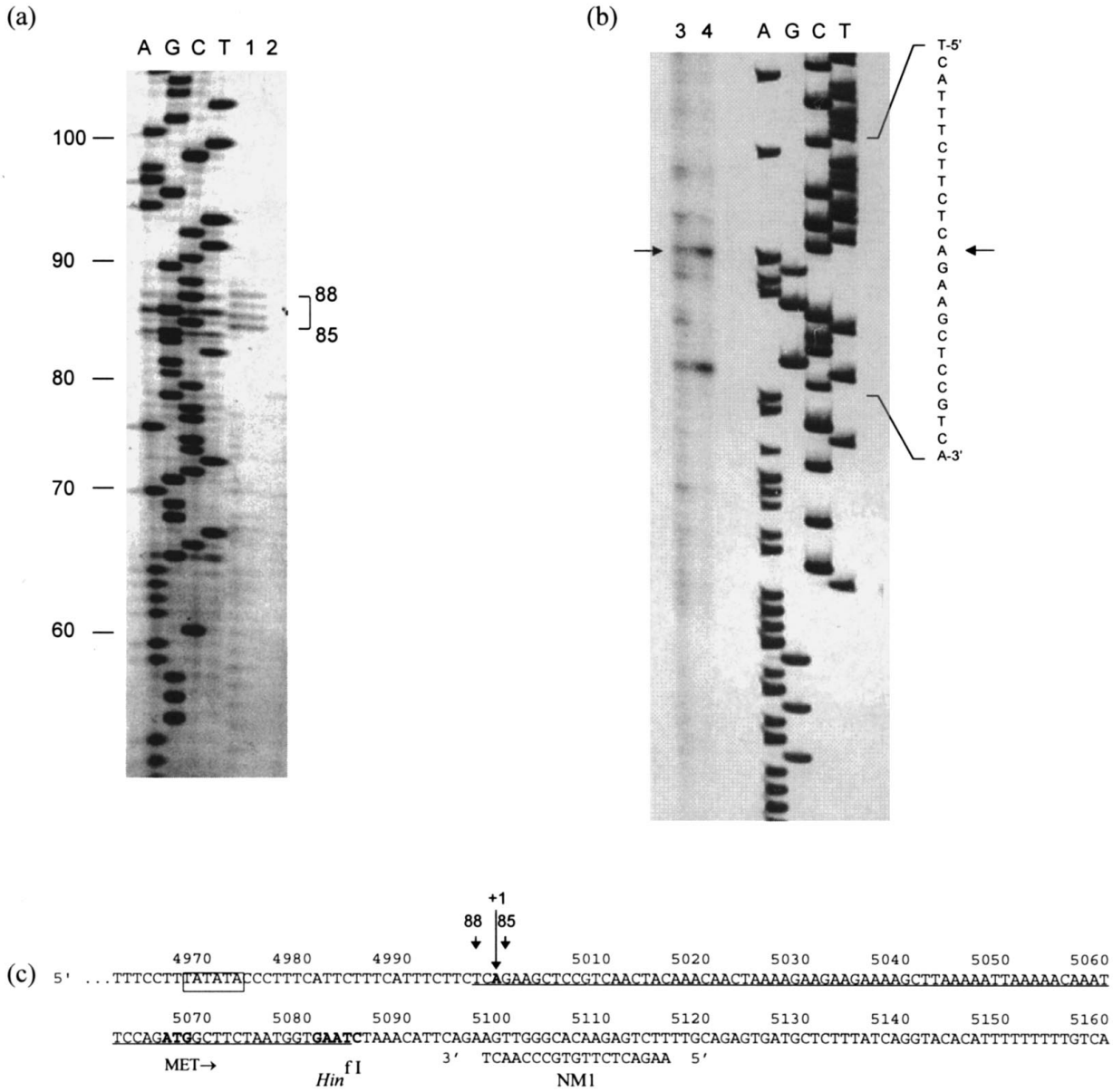

Figure 4. Assignment of the CCoAOMT transcription start site. (a) S1 mapping. A 213 bp genomic fragment ranging from −197 (*Dpn*I) to +16 (*Hinf*I) relative to the translation start site was hybridized with 1 μg (lane 1) or 10 μg poly(A)$^+$ RNA (lane 2) from elicited parsley cells and the remaining fragments after S1 nuclease digestion were analysed on a 6% sequencing gel by comparison to a sequencing ladder of pBluescript II template (Stratagene) with Reverse Primer (5′-CAGGAAACAGCTATGAC-3′). (b) Primer-extension assay with ^{32}P-labelled oligonucleotide NM1 and 2 μg of individual batches of poly(A)$^+$ RNA (lane 3 and 4) from elicited parsley cells. The reaction products were analysed on a 6% sequencing gel and compared to a sequencing ladder that resulted from sequencing the genomic *Ava*II fragment (Fig. 2) with primer NM1. One of the two major products corresponding to the S1 nuclease results is indicated by an arrow. (c) Schematic outline of the results from S1 mapping and primer-extension analysis. The DNA fragment protected in the S1 nuclease assays is underlined. The position of the transcription start site (+1), corresponding to the upper primer-extension product, is indicated by an arrow. The position and sequence of the primer NM1 is depicted.

213 bp genomic template spanning the region from –197 (*Dpn*I) to +16 (*Hin*fI) (Fig. 4) relative to the start of translation. These assays assigned 4 consecutive nucleotides as potential transcription start sites, and the correct nucleotide was identified by complementary primer-extension analysis at 67 bp upstream of the coding region (Fig. 4). A second, much shorter primer-extension product (Fig. 4) did not correspond to the results from S1 nuclease protection assays and must have resulted from premature transcription termination. The designated transcription start was preceded by a 'TATA' box 31 bp further upstream (Fig. 3), and the sequence context complies with that of other plants [20]. Nuclease protection assays with an *Nco*I template (+1 to +522) from the cDNA [49] eventually confirmed the mismatch of 5′-UTR and the genomic sequence beyond 11 bp upstream of the translation start site (data not shown). Subsequent data bank alignments with more recently released sequences suggest that, in the course of cDNA cloning, an incomplete CCoAOMT cDNA transcript had been ligated with a plastid targeting signal [29].

Copy number of CCoAOMT genes

Southern blot analysis was carried out to specify the number of CCoAOMT genes in the parsley genome. DNA isolated from parsley leaves was digested with either *Eco*RV, *Hin*dIII or a combination of *Eco*RI and *Eco*RV, and the electrophoretically separated fragments were blotted for hybridization. The labelled genomic 2.6 kb *Hin*dIII fragment spanning the full coding region of the CCoAOMT gene (Fig. 2) was used as the hybridization probe. Based on the restriction map (Fig. 2), two gene fragments of roughly 4.8 and >5.8 kb were expected upon digestion with *Eco*RV or of 4.7 and 3.6 kb from the combined restriction with *Eco*RV and *Eco*RI; restriction with *Hin*dIII should yield only one fragment of 2.6 kb. Most of these fragments were verified in the Southern experiments (Fig. 5), although the bands expected at >5.8 and 3.6 kb were not observed; instead, single bands hybridized at 4.8 and 4.7 kb (Fig. 4). The bands at >5.8 and 3.6 kb appeared after prolonged autoradiographic exposition or less stringent washing conditions (not shown) indicating a weak base pairing, since the labelled DNA probe spanned only a minor portion of these sequences, while the appearance of additional bands could reflect the presence of different alleles. Overall, the results suggest that the parsley CCoAOMT is encoded by only two genes, which might represent the allelic forms of one gene.

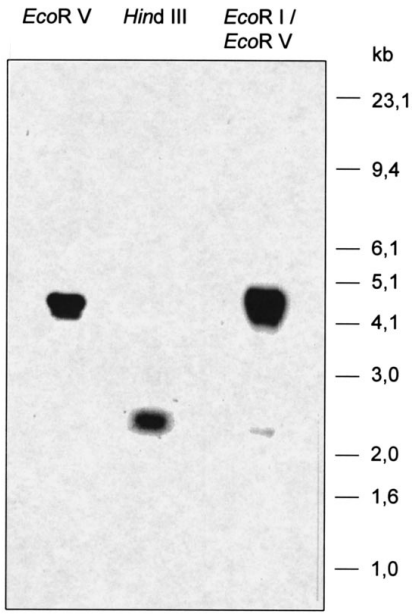

Figure 5. Southern hybridization of genomic parsley DNA. Genomic DNA (10 μg) was digested with either *Eco*RV, *Hin*dIII, or *Eco*RI/*Eco*RV, separated on a 0.7% agarose gel, and blotted onto nitrocellulose filters. The genomic 2.6 kb *Hin*dIII fragment spanning the complete CCoAOMT coding region (Fig. 2) was used as a hybridization probe.

Consensus regulatory promoter elements

Alignment studies of the 1.5 kb 5′-flanking region revealed the presence of several *cis*-regulatory elements in the CCoAOMT gene. Besides the 'TATA' box (–26 to –31), two putative 'CAAT' boxes (–122 to –127 and –191 to –196) were recognized (Figs. 2 and 3). Furthermore, a tandem repeat of 14 bp, spanning the regions –1252 to 1265 and –1277 to –1290, is incompletely repeated from -691 to -704, and a reverted copy of the SV 40 enhancer core sequence GTGGA/T A/T A/T [54] is found at –341 to –347 (Figs. 2 and 3). Such enhancer sequences were reported previously from the promoters of *Phaseolus vulgaris* phenylalanine ammonia-lyase genes, PAL2 and PAL3 [4]. Inverted repeats of 9 and 8 bp, respectively, were also present in the CCoAOMT promoter (–228 to –236 and –345 to –353, –376 to –383 and –842 to –849), the former of which partly overlaps with the SV 40-like enhancer element (Fig. 3). In addition, the gene contains two palindromic sequences in close proximity to the transcription start site (–57 to –48 and –6 to +5), the latter showing an interrupted dyad symmetry. Moreover, the promoter harbors two 12 bp (–288 to

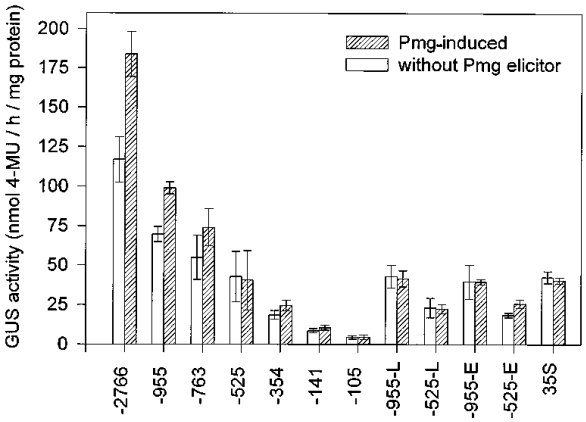

Figure 6. Elicitor-inducible transient expression of CCoAOMT promoter-GUS fusions in parsley protoplasts. The length of the 5′ unilaterally deleted promoter fragments derived from a −2766 to +40 promoter fragment is shown on the bottom. Transformation with GUS construct lacking the CCoAOMT promoter resulted in negligible GUS expression. The deletion of the L or E box in the −955 and −525 constructs, respectively, is indicated by the suffix minus L or E. The 35S/GUS construct (pRT99/GUS [19]) was used as control. Values are the average of eight independent experiments. 4-MU, 4-methyl-umbelliferone.

–277 and –118 to –107) and one 6 bp consensus elements (–591 to –586) which appear to be conserved among the enzymes of the inducible phenylpropanoid pathway and at a similar spacing to the transcription start site [31]. These elements had been detected previously by *in vivo* footprint studies in the promoter of parsley phenylalanine ammonia-lyase and designated box P, L, and A [32]. The elements were advanced as *cis*-regulatory elements involved in the transcriptional induction of genes responding to stimuli such as fungal elicitation and UV-light irradiation [13, 31, 32].

Promoter deletion analysis

The definition of promoter regions that are specifically required for transcriptional induction of the CCoAOMT gene and the assignment of the minimal elicitor-responsive promoter can be achieved by transient expression assays of promoter-GUS constructs. Parsley protoplasts are particularly suitable for these assays, since they retain the responsiveness to UV light or elicitor stimulation [6]. An extended CCoAOMT promoter (–2766 to +40) was successively truncated from the 5′-terminus, and parsley protoplasts were transformed with the corresponding promoter-GUS constructs [6]. GUS activity was monitored fluorometrically after treatment of the protoplasts with fungal elicitor (*Pmg*) (Fig. 6). The full-size promoter-GUS construct showed high elicitor inducibility, but the expression efficiency and the responsiveness decreased with each successive deletion, and the latter was fully lost upon reduction to 141 bp and below. The induction of activity driven by the full-size promoter in these experiments was only about two-fold, which made the interpretation of inducibility with the truncated 525 to 354 bp promoter-constructs rather difficult. Nevertheless, the –955 promoter-GUS construct reliably retained its elicitor responsiveness (Fig. 6) and was employed for further deletion analysis. In addition to box L, which had been described as a *cis*-regulatory element from genes of the inducible phenylpropanoid pathway [15, 16, 32], a novel sequence motif, box E, was identified upstream of box L in the –955 promoter by *in vitro* DNA-protein binding (see below). Deletion of either box E or L from the promoter resulted in the complete loss of elicitor-inducibility (Fig. 6) and thus confirmed their function as *cis*-regulatory elements. These two boxes, however, are closely spaced on the promoter sequence (Figs. 2 and 3), and deletion of one box likely affects *trans*-factor-binding of the neighbouring box.

In vitro *analysis of DNA-protein interaction*

The promoter of –354 to +40 bp contains the P and L boxes previously proposed as *cis*-regulatory elements for stress-regulation [31, 32]. This promoter was shortened to yield the P/L-probe, spanning the sequence −354 to −52 and still containing the P and L boxes. This probe was employed to study *in vitro* the interaction with nuclear proteins. The radioactively labelled DNA was incubated with nuclear protein extracted from either uninduced or elicitor-induced cultured parsley cells, and the binding interaction was monitored by electrophoretic mobility shift assays (EMSA). Nuclear protein from the elicited cells induced three shifted bands, and the specificity of this interaction was proven by binding competition with the unlabelled P/L probe (Fig. 7). Unrelated DNA such as pBluescript (Stratagene) or the 35S promoter [19] failed to reverse the binding to a significant extent. Furthermore, nuclear protein from uninduced control cells induced shifts of lower intensity. Thus, nuclei from elicited parsley cells contain protein(s) that interact specifically with the P/L probe, and these *trans*-active factors are induced in the nuclei upon elicitation.

In order to pinpoint the DNA binding motif, additional *in vitro* DNase I footprint studies were carried out with the P/L probe. These studies revealed that the

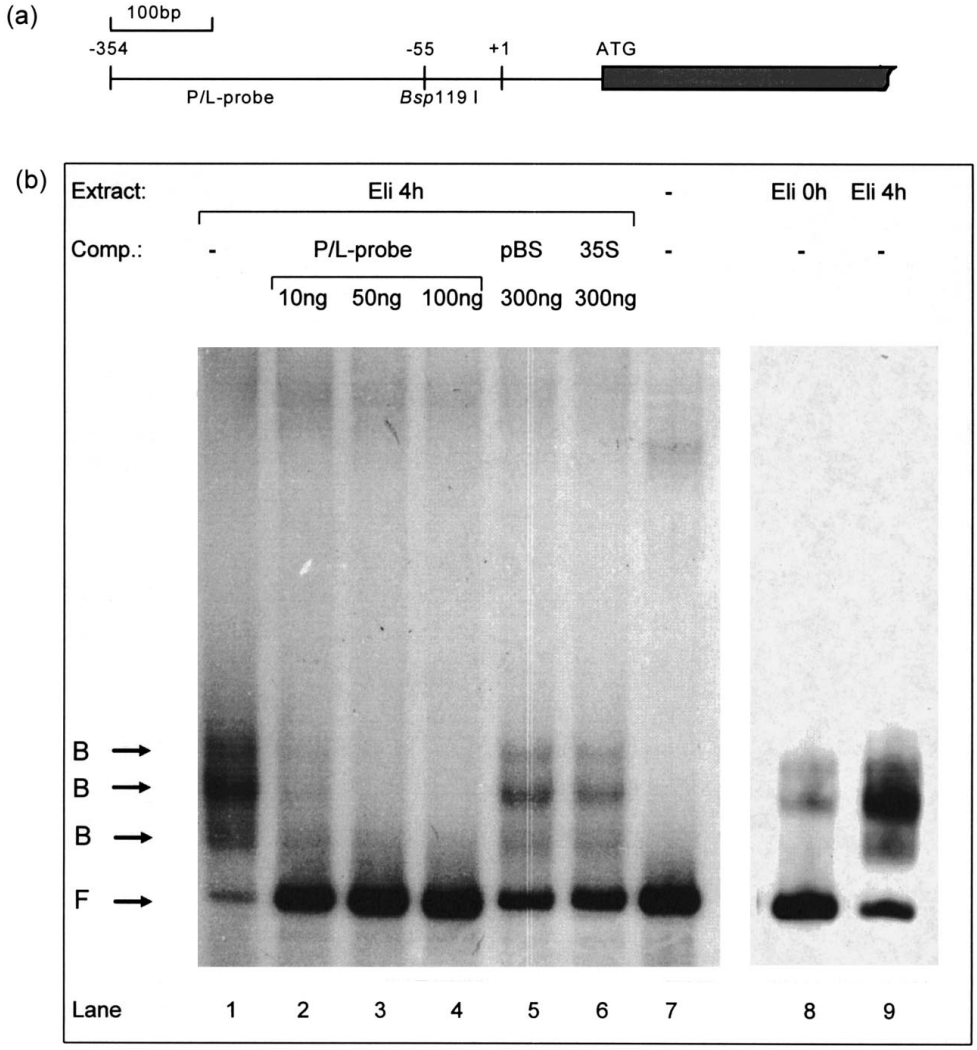

Figure 7. Interaction of nuclear proteins from parsley cell cultures with the CCoAOMT promoter. (a) −354 to −52 CCoAOMT promoter fragment containing boxes P and L (P/L probe) used for electrophoretic mobility shift assays (EMSA). (b) Binding assays with nuclear extracts from parsley cells treated with *Pmg* elicitor for 0 h (lane 8) or 4 h (lane 1 and 9). Binding competition was carried out by the addition of 10, 50 and 100 ng, respectively, of unlabeled P/L probe (lanes 2, 3 and 4). Alternatively, 300 ng pBS plasmid (lane 5) or CaMV35S promoter DNA (lane 6) was employed. The labelled P/L probe was incubated for control in the absence of nuclear protein (lane 7). F, unbound P/L probe; B, specific promoter-protein complexes.

nuclear extract from elicited parsley cells specifically protected a 12 bp motif in the P/L probe from DNase I digestion, whereas nuclear extracts from uninduced cells again showed a much weaker effect (Fig. 8). This 12 bp *cis*-element is described here for the first time and is designated in the context of this work as box E. Binding of nuclear protein to this particular element was verified by the synthesis of a 33 bp oligonucleotide probe, containing the box E, and the use of this labelled oligonucleotide in EMSA assays (Fig. 9). Binding specificity was corroborated by competition with the unlabeled oligonucleotide (Fig. 9). Furthermore, the oligonucleotide was capable of competing with the P/L probe in binding assays (Fig. 10), supporting the identification of the footprint. Unexpectedly, boxes P and L, which had been assigned from *in vivo* footprint analysis [31, 32], did not interact in the *in vitro* binding studies with the P/L probe (exemplified for box L in Fig. 8). The results suggest that binding of nuclear protein to box E is specific and independent

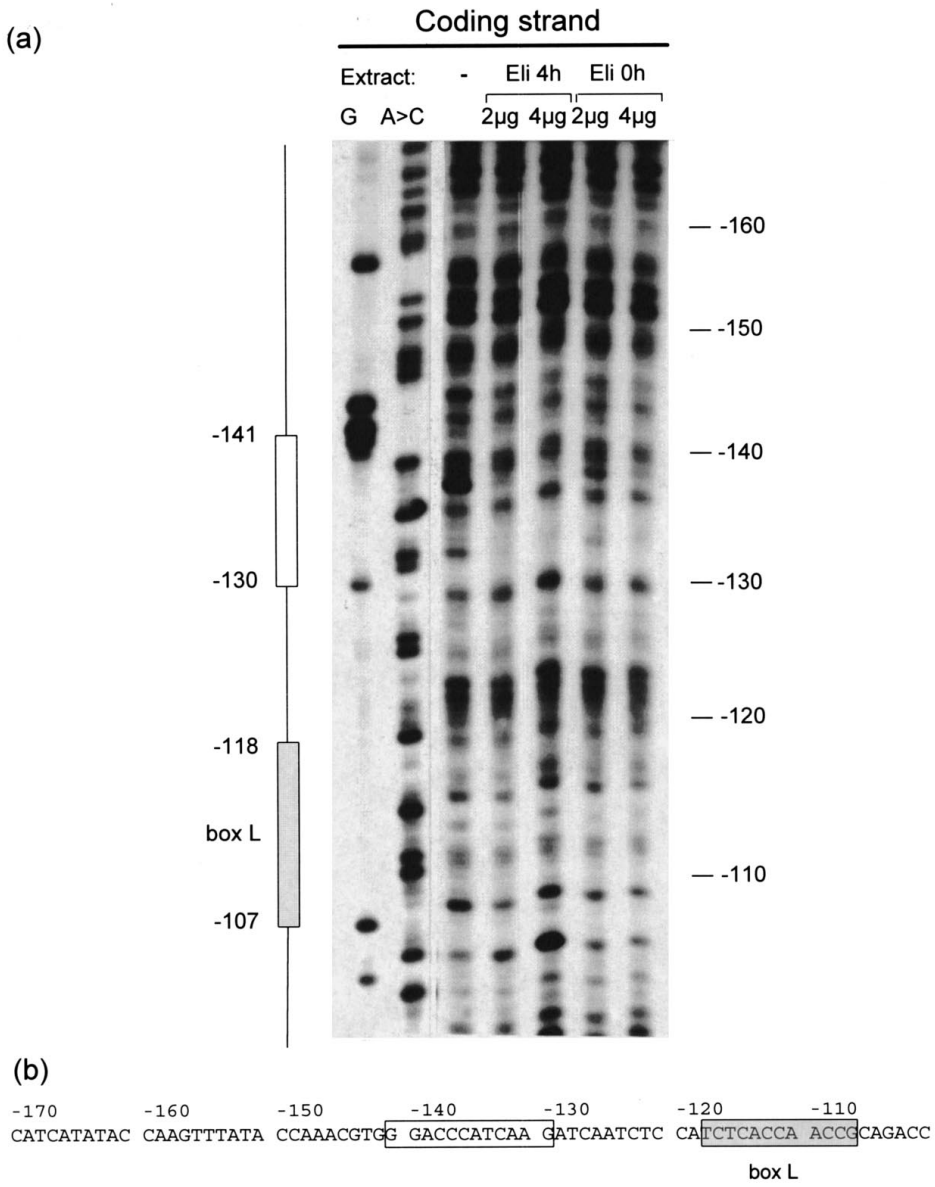

Figure 8. DNase I footprint analysis of the CCoAOMT promoter region from −354 to −52 (P/L probe). (a) Section of the promoter footprint pattern. The ^{32}P-labelled P/L-probe was incubated with nuclear extracts from parsley cells which had been treated with *Pmg* elicitor for 0 h or 4 h (Eli 0 h, Eli 4 h) and digested with DNase I. The region protected from DNase I digestion in the presence of nuclear protein is designated with an open box, the position of the L box is given by a shaded box. G- and A>C-specific Maxam-Gilbert reactions of the same fragment indicate the position of the protected region. (b) Sequence of the depicted promoter region including the L box and the protected region.

of the DNA superstructure, whereas binding of *trans*-active factors to boxes P and L may require additional prerequisites like a specific *in vivo* folding of DNA.

Making use of an *Ava*II restriction site located in the footprint, the P/L probe was digested, and the individual restriction fragments (α- and β-probe) were employed for competition in the *in vitro* binding studies (Fig. 10). Neither fragment competed successfully with the P/L probe for binding, and, therefore, binding of the *trans*-active factors from the nuclei of elicited parsley cells requires either the complete box E or the nucleotides immediately neighbouring to the restriction site.

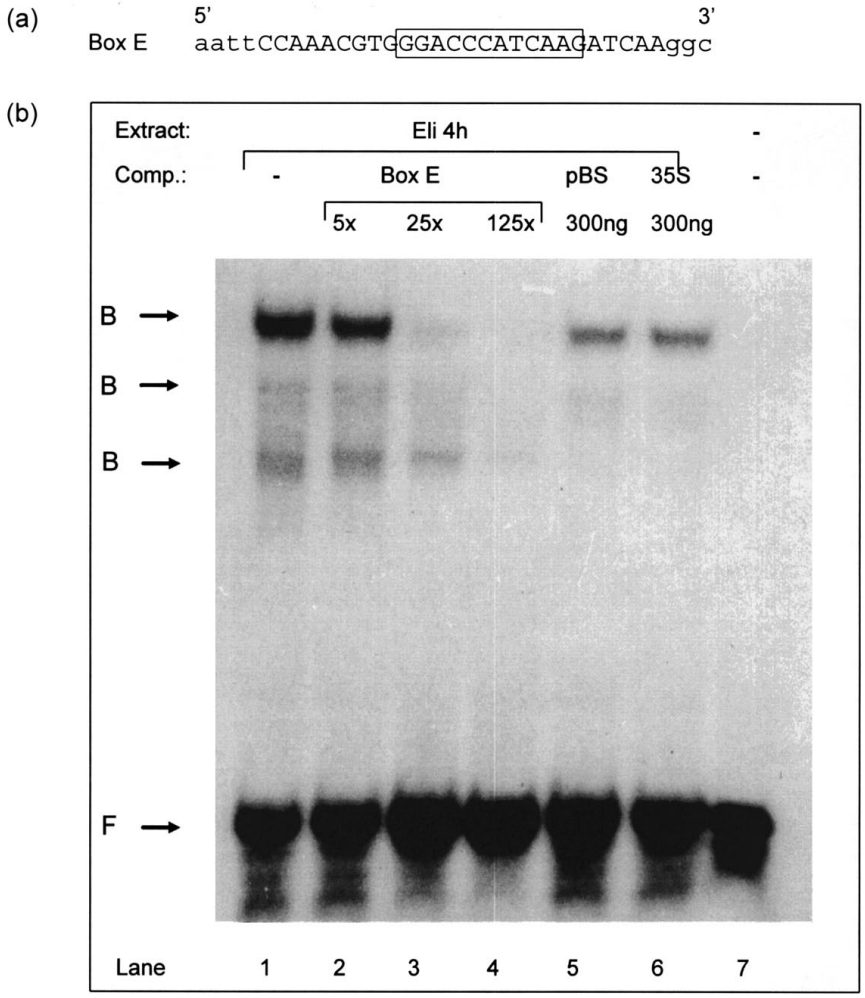

Figure 9. Interaction of proteins from parsley nuclear extracts with box E. (a) Synthetic box E probe used for the electrophoretic mobility shift assays (EMSA). Nucleotides in capital letters correspond to the promoter sequence. The region which was protected in DNase I footprint analysis (Fig. 8) is boxed. (b) Binding of nuclear factors from parsley cells that had been treated with *Pmg* elicitor for 4h (lane 1) to labelled box E (3.8 fmol, 20 000 dpm/lane). Binding competition was carried out by the addition of increasing amounts of unlabelled box E oligonucleotide (lanes 2, 3 and 4) or with non-specific DNA (lane 5 and 6). The labelled box E was incubated for control in the absence of nuclear protein (lane 7) F, unbound box E probe; B, specific complexes

Since the box E appeared to be a novel *cis*-regulatory element among the genes of the elicitor-responsive phenylpropanoid pathway, the available sequence information for the corresponding genes was screened for such a motif. Obviously, homologous motifs are conserved in the other genes and are positioned from –320 to –54 from the start of transcription (Fig. 11). An oligonucleotide of 32 bp covering the putative box E from the parsley phenylalanine ammonia-lyase gene, PAL1, was synthesized and binding of nuclear factors as well as competition experiments with boxes E or L from the CCoAOMT gene were carried out (Fig. 12). For these experiments, the 33 bp oligonucleotide corresponding to the CCoAOMT box E and a 30 bp oligonucleotide representing the CCoAOMT box L were synthesized. Clearly, the PAL1 box E was capable of binding nuclear factors from the elicited parsley cells, and the CCoAOMT box E competed with this interaction, although less efficiently. Considering the small size of box E, however, one has to be aware that homologous motifs (8 matches out of 10) can be recognized by data bank alignment in a number of unrelated genes, and the transcriptional relevance has to be proven in each instance. Surpris-

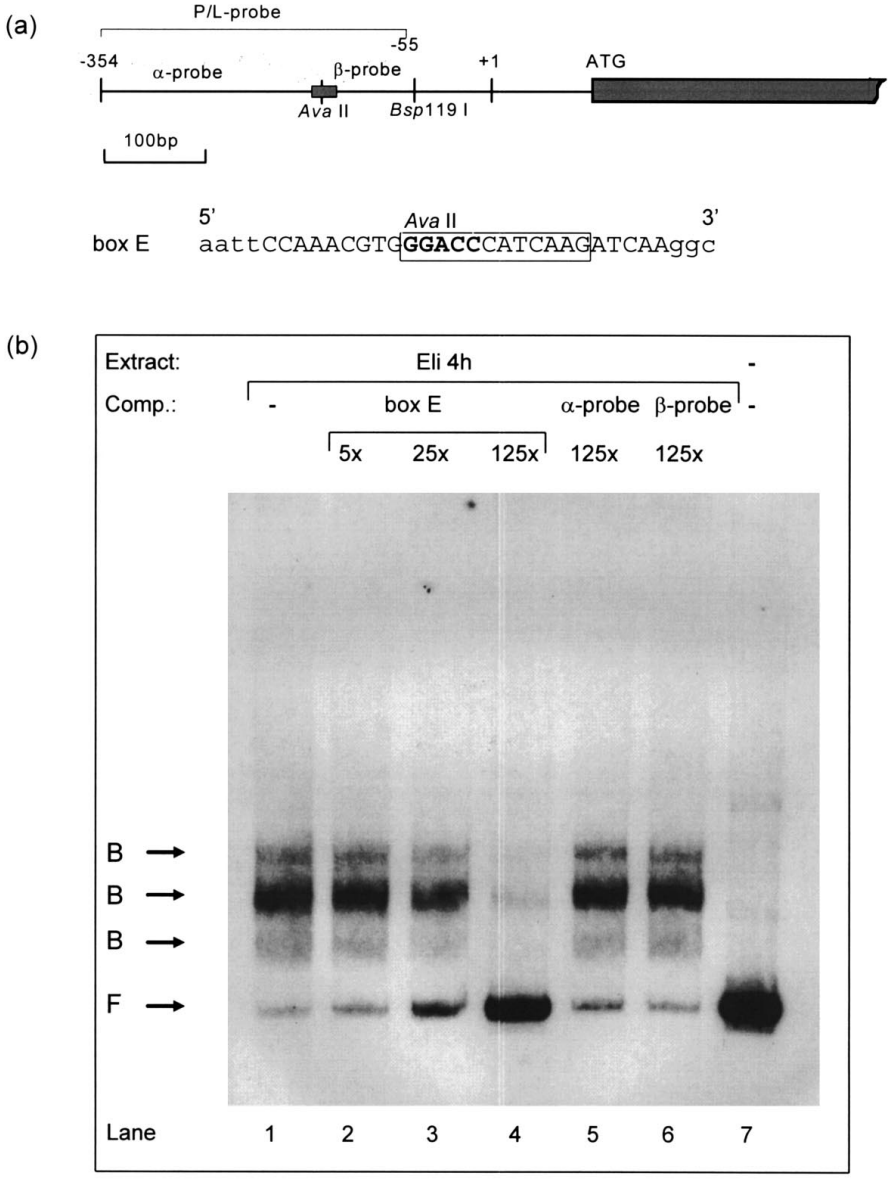

Figure 10. Binding competition of labeled CCoAOMT promoter fragment (P/L probe) with box E oligonucleotide. (a) P/L probe used to analyse competition of box E for binding. Competitor DNA α and β probes were generated through *Ava*II (-141) cleavage of the P/L probe (-354 to -52). The *Ava*II restriction site (GGWCC) is located in the identified footprint (filled box in the schematic outline of the CCoAOMT promoter or open box in the corresponding sequence). (b) The ^{32}P-labelled P/L probe (1,5 fmol, 20 000 dpm/lane) was incubated with nuclear extracts from elicited parsley cells. Competition was carried out with the indicated molar excess of unlabelled box E oligonucleotide (lanes 2, 3 and 4) or α and β probe.

ingly, competition experiments with CCoAOMT box L revealed a complex pattern (lane 5, Fig. 11), which resulted in the decreased intensities of two bands and an enhanced intensity of the third band. This effect is puzzling, since no nuclear factor binding of the CCoAOMT box L had been observed in the *in vitro* footprint assays with the P/L-probe, and this pattern suggests that protein-protein interaction might interfere with the protein-DNA-binding.

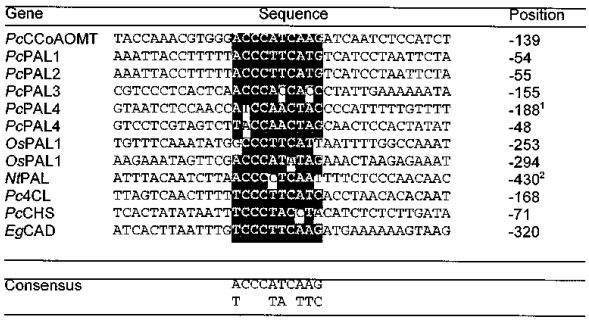

Figure 11. Conserved box E homologous motifs in promoters of plant phenylpropanoid genes. The darkened sequences represent the DNase I footprint in the CCoAOMT promoter, as well as corresponding promoter sequences of other phenylpropanoid genes. The position of the first 5′ consensus nucleotide relative to the transcription start site is indicated. Pc, *Petroselinum crispum* [32, 33]; Os, *Oryza sativa* [39]; Nt, *Nicotiana tabacum* (GenBank Accession number M84466); Eg, *Eucalyptus gunnii* [10]; PAL, phenylalanine ammonia-lyase; 4CL, 4-hydroxy-cinnamate CoA-ligase; CHS, chalcone synthase; CAD, cinnamoyl alcohol dehydrogenase. [1] Partially overlapping the box P sequence of PAL1 [32]. [2]Position relative to the translation start site.

Discussion

The coding region of the parsley CCoAOMT gene (Fig. 3) matched the sequence elaborated previously for the cDNA from elicited parsley cell cultures [49]. This included the 3′-flanking region down to the polyadenylation site, AATATA (from +1566 to +1571; Fig. 3), as well as the sequence context of the translation start site in which the consensus G at position +4 is conserved while at –3 (Fig. 3) C replaces the more common A [20]. The mosaic composition of the open reading frame is common in plant genes and has been reported, for example, for 4-coumarate:CoA ligase, Pc4CL-1 and Pc4CL-2, from parsley [33]. The intron interposition may even enhance the gene expression of some genes; such an enhancement was proposed to be independent of the promoter activity, but seems to depend delicately on the border sequences of the exonic regions [34].

The sequence homology of the CCoAOMT gene and the cDNA, however, ended 11 bp upstream of the translation start codon (Fig. 3). Primer extension analysis and S1 nuclease protection assays with the genomic template (Fig. 4) revealed instead the transcription start site 67 bp upstream of the translation start, irrespective of the fact that the sequence does not strictly follow the Py–Py A PyPyPy motif suggested for a transcription start consensus [3]. Thus, the 5′-UTR sequence reported for the cDNA [49] has to be revised. A data bank search for the 5′-UTR mismatch cDNA region revealed close homology to a plastidic inner envelope target sequence from pea (GenBank, accession number M73744) that was registered after the cDNA had been isolated [29]. The information available suggests that incomplete copies of plastid target and CCoAOMT transcripts were ligated during the cDNA subcloning. Fusion of unrelated transcripts has been observed frequently during cDNA cloning and seems to be an immanent problem when long transcripts are selected [23]. Southern blot hybridization suggested that the CCoAOMT is encoded by only two genes in the parsley genome, which might represent allelic forms of one gene. Parsley PAL, in contrast, is encoded by four genes, and at least one of these is differentially regulated in response to environmental stresses or in different tissues [31].

Several putative *cis*-regulatory elements were pointed out in the CCoAOMT promoter by sequence alignment. Although their factual relevance has yet to be confirmed, the boxes A, P and L (Figs. 2 and 3) deserve particular attention. These motifs had initially been identified by *in vivo* footprint experiments as light- and elicitor-responsive promoter elements in the parsley *Pc*PAL-1 and *Pc*4CL genes [16, 32]. They were later found to be present in various other genes of the phenylpropanoid pathway, i. e. in PAL and 4CL genes from other plants, in chalcone synthase genes from different sources as well as in a CAD gene from *Eucalyptus gunnii* [10, 15, 28, 31, 41], and, accordingly, classify CCoAOMT as a typical enzyme of the elicitor-inducible phenylpropanoid pathway. Moreover, the relative position of these elements in the promoter appears to be important for the mode of gene expression and has been studied scrutinously in case of the parsley PAL genes. In three of the four PAL genes (PAL1, PAL2 and PAL3) the P box is positioned upstream followed in sequential order by the A and L boxes; the expression of these genes is inducible by light irradiation, elicitor treatment or wounding of the tissue. The fourth gene (PAL4) contains the L-box far upstream followed by the P and A boxes; this gene is not expressed upon light irradiation [31]. The 4CL genes in parsley contain yet another spatial pattern of the P, A and L boxes, but are regulated similarly to PAL1-3 [31]. In spite of the convincing functional relevance, transient expression assays with promoter-reporter gene constructs in parsley protoplasts demonstrated that these *cis*-active elements, either alone or in combination, are not sufficient to explain the responsiveness to light or elicitor

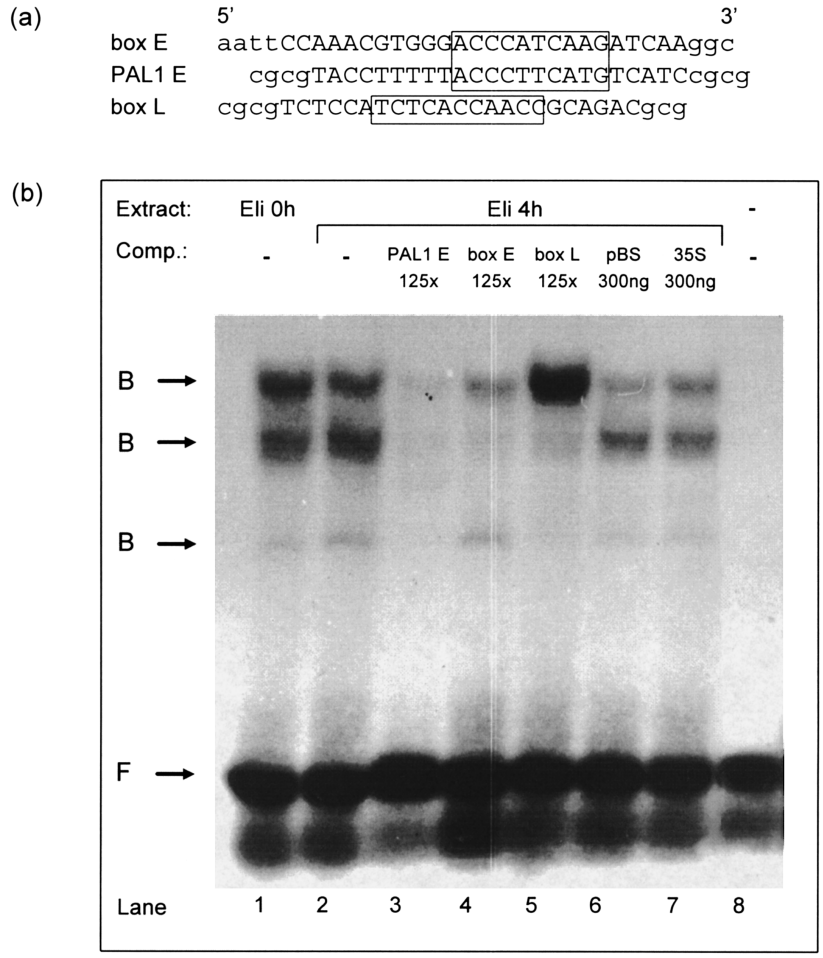

Figure 12. Binding and competition experiments of parsley nuclear factors to the element corresponding to box E in the PAL1 promoter (PAL1 E). (a) Oligonucleotide probes used in this analysis. Nucleotides in capital letters indicate the corresponding promoter sequence. Box E and box L are elements from the parsley CCoAOMT promoter. (b) Nuclear extracts from parsley cells, which were induced with Pmg elicitor for 0 h (lane 1) or 4 h (lane 2–8). were incubated with labeled PAL1-E oligonucleotide (3.5 fmol, 20 000 dpm/lane). The indicated competitors were used at a 125-fold molar excess or 300 ng in the case of pBS plasmid and CaMV35S promoter DNA.

[31], and the combinatorial interaction of these boxes even with exonic sequences has been suggested [15].

A relatively short promoter sequence (−354 to −52 bp) was used for *in vitro* footprint studies. Both the P and the L box were retained in this sequence, which was therefore assigned as the P/L probe (Fig. 7). Gel shift experiments with this DNA demonstrated the elicitor-responsive binding of nuclear protein(s) (Fig. 7) and thus suggested the transcriptional activation by *trans*-active factor(s) binding. The results are reminiscent of the results reported previously for the regulation of PAL and 4CL genes [32]. It should be noted, however, that the results of *in vitro* footprint experiments cannot be directly related to those of *in vivo* footprinting, and both types of studies have their particular limitations. As an example, an elicitor-induced *trans*-active factor binding was shown in gel shift experiments for box L but not box P of *Pc*PAL1 gene, although the relative transcript abundance for the box P-binding factor was transiently induced by elicitor treatment of parsley cells [5, 13]. In our *in vitro* studies, binding of nuclear factor(s) to the labeled, truncated (–354/–52) parsley CCoAOMT promoter occurred to a sequence motif adjacent to box L rather than to box P or box L (Fig. 8). This footprint, designated box E, appears to be capable of binding several protein factors (Fig. 9), and the interaction of the P/L probe with nuclear proteins could be suppressed by competition with

excess non-labelled box E oligonucleotides (Fig. 10). Furthermore, binding of the *trans*-active factors from the nuclei of elicited parsley cells requires either the complete box E or the nucleotides immediately neighbouring to the *Ava*II restriction site located in the footprint box E (Fig. 10). It has been reported for other *cis*-regulatory elements that four of six nucleotides are sufficient to explain the specific interaction with nuclear proteins [56]. The fact that box L did not bind nuclear protein in the *in vitro* footprint studies suggests that binding of *trans*-active factors to box L (and likely also to box P) requires the *in vivo* conformation of the DNA. Moreover, our results suggest that binding of nuclear factors to the novel box E may precede the binding to boxes P and/or L.

The transient expression assays conducted with the CCoAOMT promoter-GUS constructs in parsley protoplasts clearly confirmed that sequence motifs in addition to the P, A and L boxes are required for the full responsiveness to elicitor treatment and a high rate of expression. For example, deletion of 5′-regions that did not affect the P, A or L-box resulted in a pronounced reduction of the elicitor-induced GUS expression, which may have been due to the loss of enhancer sequences (Fig. 6). Most notably, however, deletion of either box E or box L from the elicitor-responsive promoter significantly reduced the expression efficiency and abolished the responsiveness to elicitor. These two motifs are aligned in close distance in the promoter sequence and appear to affect mutually their binding of *trans*-active factors. The deletion experiments clearly support the role of the newly described box E as an elicitor-responsive, *cis*-regulatory element.

The significance of box E as a novel *cis*-regulatory element is further supported by the identification of similar sequences in the promoters of other phenylpropanoid genes such as PAL, 4CL, CHS and CAD (Fig. 11). For instance, a motif with high homology to box E is present in the parsley 4CL promoter (position –160 to –168), and this motif among others was proposed as a *cis*-regulatory element on the basis of two independent *in vivo* footprint studies [2, 16]. The deletion of a promoter region in the 4CL promoter that encompasses both the box E and box L resulted in a striking reduction of promoter activity in parsley protoplasts or transgenic tobacco, and in these studies, further deletions of upstream DNA without any obvious regulatory elements led to an even steeper decline in promoter activity [15]. Similar observations were made with the CCoAOMT promoter (Fig. 6). In another set of experiments the motif representing box E in parsley PAL1 and PAL2 promoters was synthesized and tested for its binding and competition capacities (Fig. 12). This motif specifically bound proteins from parsley nuclear extracts, and the interaction could be displaced by the CCoAOMT box E oligonucleotides. In summary, the results suggest that the box E described for the first time in this report is also contained in the promoters of other genes of the inducible phenylpropanoid pathway. Our results suggest that box E is involved in the transcriptional regulation upon elicitation of plant cells, and binding of *trans*-active factors to this box seems to occur at an early stage of regulation. The relevance of this novel *cis*-regulatory element is under investigation.

Acknowledgements

We are indebted to Bayer AG, Forschungszentrum Monheim, for the opportunity to collaborate and particularly to Professor D. Berg, Dr R. Hain and Dr J. Reif for kindly hosting one of us (B.G.) for several months in their laboratory and providing support and valuable advice. We also thank our colleagues J. Schröder, R.E. Kneusel, U. Mühlenhoff and Q. Yang for helpful suggestions and critical reading of the manuscript. The work described in this report was supported by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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