

Molecular Cloning and Expression of 4-Coumarate:Coenzyme A Ligase, an Enzyme Involved in the Resistance Response of Soybean (*Glycine max* L.) against Pathogen Attack¹

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We have isolated three classes of cDNAs that probably encode three 4-coumarate:coenzyme A ligase (4CL) isoenzymes in soybean (*Glycine max* L.). The deduced amino acid sequences reveal several regions of extended sequence identity among 4CLs of all plants analyzed to date. The sequences of two of these regions are consistent with a domain structure proposed for a group of enzymes catalyzing the ATP-dependent covalent binding of AMP to their substrates during the reaction sequence. By using two cDNA fragments that do not cross-hybridize under the conditions used, we demonstrate that 4CL in soybean is very likely encoded by a small gene family. Members of this family are differentially expressed in soybean cell cultures treated with β -glucan elicitors of *Phytophthora megasperma* f. sp. *glycinea* or in soybean roots infected with either an incompatible or compatible race of the fungus. These results are in agreement with our previous observation that elicitor treatment of soybean cells caused a preferential enhancement in the activity level of one of the 4CL isoenzymes. In soybean, 4CL isoenzymes possessing different substrate affinities for substituted cinnamic acids, and showing differential regulation to environmental stress, may play a pivotal role in distributing substituted cinnamate intermediates at a branch point of general phenylpropanoid metabolism into subsequent specific pathways.

Phenylpropanoid derivatives of higher plants constitute a group of compounds with large structural and functional diversity. Phenylpropanoids have been proposed to serve as flower pigments, UV protectants, defense chemicals (phytoalexins, insect repellents), allelopathic agents, and signal molecules in plant-microbe interactions. Furthermore, phenylpropanoids are the building units of polymeric support structures such as lignin or lignin-like cell-wall components and of soluble or wall-bound phenolics (Ebel and Hahlbrock, 1982; Hahlbrock and Scheel, 1989).

The synthesis of many of the phenylpropanoid compounds involves the reactions of general phenylpropanoid metabolism that are catalyzed by Phe ammonia-lyase, cinnamate

4-hydroxylase, and 4CL. The products of this reaction sequence, CoA thiol esters of 4-coumaric acid and other substituted cinnamic acids, are substrates for subsequent individual pathways. It has been suggested that 4CL plays a pivotal role at a branch point in phenylpropanoid metabolism by regulating the flux of substituted cinnamate intermediates into subsequent products. Such a role is supported by the existence of isoforms of 4CL with different substrate affinities and tissue distribution in several leguminous and woody plants, including soybean (Knobloch and Hahlbrock, 1975), pea (Butt and Wilkinson, 1978), petunia (Ranjeva et al., 1976), poplar (Grand et al., 1983), and oat (Knogge et al., 1981). In contrast, the two 4CL isoforms from parsley have very similar molecular properties, as do those of potato, and show no evidence of differential expression in response to various types of stimulus imposed on whole plant tissues and cultured cells (Douglas et al., 1987; Lozoya et al., 1988; Becker-André et al., 1991). It therefore appears unlikely that the 4CL isoforms in these plants have differential roles in phenylpropanoid branch pathways.

In soybean (*Glycine max* L.), challenge with the fungal pathogen *Phytophthora megasperma* f. sp. *glycinea* or treatment with an elicitor derived from the fungal cell walls causes a profound activation of phenylpropanoid metabolism as part of the plant's defense. Among the compounds that accumulate close to infection sites or in elicitor-treated cells are pterocarpan phytoalexins (Ebel and Grisebach, 1988), isoflavone conjugates (Graham and Graham, 1991b; Morris et al., 1991), and wall-bound phenylpropanoid compounds (Graham and Graham, 1991a). Induction of phytoalexin accumulation in soybean involves a whole complement of enzymes of the biosynthetic pathway (Ebel and Grisebach, 1988) and is preceded by transient increases in the activities of enzymes of general phenylpropanoid metabolism including 4CL, the flavonoid-isoflavonoid pathway, and the pterocarpan-specific branch. Earlier experiments indicated that of the two isoenzymes of 4CL identified (Knobloch and Hahlbrock, 1975), only the activity of isozyme 2, for which a specific involvement in flavonoid biosynthesis has been

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Abbreviations: CHS, chalcone synthase (EC 2.3.1.74); 4CL, 4-coumarate:CoA ligase (EC 6.2.1.12); IPTG, isopropyl β -D-thiogalactoside.

postulated, was significantly enhanced following elicitor treatment of soybean cell cultures (Hille et al., 1982).

To elucidate the mechanisms of 4CL gene activation associated with the soybean-*P. megasperma* interaction, cDNA probes encoding 4CL were generated from elicitor-treated cell-suspension cultures. These cDNAs were characterized and used for comparative studies of the expression of 4CL transcripts in response to elicitor treatment of soybean cell cultures and in the incompatible and compatible plant-fungus interactions.

MATERIALS AND METHODS

Fungal Cultures

Phytophthora megasperma Drechs f. sp. *glycinea* Kuan and Erwin races 1 and 3 were grown as described by Ayers et al. (1976). Zoospores were obtained from 6-d-old cultures according to the method of Eye et al. (1978).

Soybean Seedlings and Infection

Soybean seeds (*Glycine max* L. Merr. cv Harosoy 63) were obtained from Dr. R. I. Buzzell (Agriculture Canada, Research Station, Harrow, Canada). Seedlings were grown under aseptic conditions on wet filter paper as described previously (Hahn et al., 1985). The taproots of 2-d-old seedlings were inoculated with a suspension of about 10^4 zoospores in 100 μ L of sterile distilled water by dip inoculation (Hahn et al., 1985). Control, uninfected seedlings were placed in 100 μ L of water.

Plant Cell Cultures and Elicitor Treatment

Soybean cell-suspension cultures (*G. max* L. cv Harosoy 63) were grown in the dark as described previously (Hille et al., 1982) and treated with *P. megasperma* elicitor (80 μ g of Glc equivalents/mL of medium) obtained by partial acid hydrolysis of purified cell walls of the fungus (Sharp et al., 1984), as described earlier (Schmidt, 1986).

Screening of a cDNA Library and Subcloning of DNA Fragments

Poly(A)⁺ RNA (5 μ g) from soybean cell-suspension cultures that had been treated with *P. megasperma* elicitor for 5 h was used for cDNA synthesis (Gubler and Hoffmann, 1983) (kit from Pharmacia Biochemicals, Freiburg, Germany). After addition of *EcoRI-NotI* adaptors, the cDNAs were ligated to *EcoRI*-digested and dephosphorylated λ gt10 DNA. Packaging was performed with a kit from Amersham Buchler (Braunschweig, Germany). Recombinant phages were selected by plating on *Escherichia coli* strain NM514 (Hoyt et al., 1982). The library was screened by using ³²P-labeled cDNA (Feinberg and Vogelstein, 1983) encoding parsley 4CL (UV16) (Douglas et al., 1987; Lozoya et al., 1988). Filters were washed as described below for northern and Southern blot analyses. Putative clones were purified by three rounds of screening, and the DNA was isolated from four purified 4CL cDNA clones.

After digestion of phage DNA with *EcoRI*, the cDNA

fragments were inserted into the *EcoRI* site of the plasmid vector pTZ19R (Zagursky and Berman, 1984). The recombinant plasmids were used to transform *E. coli* strain JM109 (Yanish-Perron et al., 1985). The largest cDNA insert (1.5 kb) was used for a further screening of the λ gt10 library.

Isolation and Analysis of RNA and DNA

Total RNA from cell-suspension cultures (3 g per assay) and excised root segments (2–2.5 g) was isolated according to the method of Langridge et al. (1982) as described by Haberer et al. (1989). For northern hybridization, 20 μ g of total RNA or 5 to 7 μ g of poly(A)⁺ RNA (Sambrook et al., 1989) was denatured and fractionated on 1.2% agarose gels containing formaldehyde (Sambrook et al., 1989). For Southern hybridization, soybean cell culture genomic DNA was prepared (Draper and Scott, 1988), and DNA samples (10 μ g) were cleaved with various restriction enzymes and fractionated by electrophoresis on 0.8% agarose. RNA and DNA gel blot analyses were performed on nitrocellulose membranes according to the method of Sambrook et al. (1989).

Blotted RNA and DNA were hybridized using ³²P-labeled parsley and soybean 4CL cDNA inserts. Prehybridization was performed at 42°C for 6 to 12 h in 24% (v/v) formamide, 5 \times SSPE, 5 \times Denhardt's solution, 0.5% (w/v) SDS, and 100 μ g/mL of denatured salmon sperm DNA. Hybridization was carried out in the same solution at 42°C for 12 to 24 h. For plaque hybridization using parsley and soybean 4CL cDNA inserts, membranes were washed two times for 1 h with 2 \times SSC, 0.5% (w/v) SDS at 60°C. For RNA blot hybridization, membranes probed with parsley 4CL cDNA insert were washed two times for 1 h with 2 \times SSC, 0.5% (w/v) SDS at 50°C, and membranes probed with soybean cDNA inserts were washed two times for 1 h with 0.1 \times SSC, 0.5% (w/v) SDS at 60°C. For genomic DNA blot hybridization, membranes were washed two times for 1 h with 0.1 \times SSC, 0.5% (w/v) SDS at 60°C.

DNA Sequence Analysis

The cDNA clones were sequenced (sequenase kit from United States Biochemical/Renner, Dannstadt, Germany) using the dideoxy chain termination method (Sanger et al., 1977). The pTZ19R system (Zagursky and Berman, 1984), helper phage M13K07 (Vieira and Messing, 1987), *E. coli* strain JM109 (Yanish-Perron et al., 1985) (all from Pharmacia, Freiburg, Germany), and the reverse sequencing primer M13/pUC (Boehringer Mannheim, Germany) were used. Suitable plasmids were obtained by subcloning cDNA fragments. Both strands of GM4CL14 and GM4CL16 were fully sequenced with overlapping clones or with the help of a synthetic oligonucleotide (19-mer). The other cDNAs were analyzed for 300 to 600 bp from both the 5' and 3' end.

Protein Expression in *E. coli*

The plasmid vector pTZ19R contained the *EcoRI* fragment GM4CL13 in the correct phase for expression of a *lacZ*/4CL fusion protein in which β -galactosidase and the *EcoRI-NotI* adaptor contributed 32 amino acids at the N terminus and

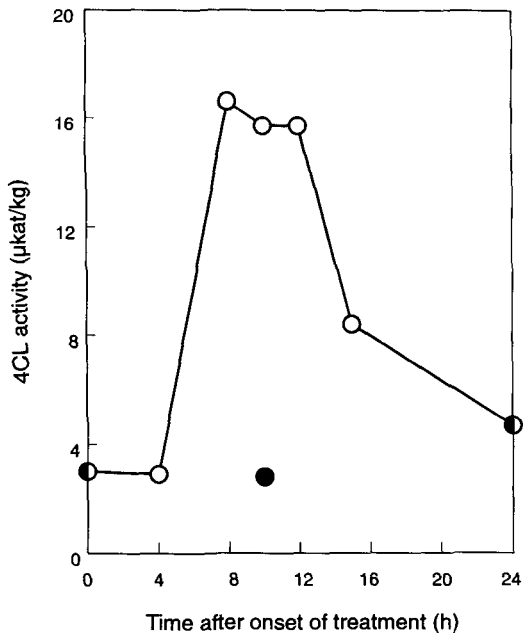


Figure 1. Changes in 4CL enzyme activity (isoenzyme 2) in soybean cell cultures following treatment with β -glucan elicitors from *P. megasperma* f. sp. *glycinea*. Cultures were exposed to elicitor solutions (O) or an equal volume of water (●) and harvested at the times shown. The activity of 4CL isoenzyme 1 remained below the detection limits of the enzyme assay during the period investigated.

GM4CL13 contributed 241 amino acids. *E. coli* strain JM109 harboring the pTZ19R/4CL plasmid was grown to an optical density of 0.8, and expression was induced by addition of IPTG to a final concentration of 1 mM. Western blot analysis was performed as described by Towbin et al. (1979) using 4CL antiserum to the enzyme from parsley (Ragg et al., 1981) and the alkaline phosphatase detection system.

Assay of 4CL Enzyme and mRNA Activity

4CL enzyme activity was measured in extracts of cell-suspension cultures (Hille et al., 1982) with the use of the photometric assay according to the method of Knobloch and Hahlbrock (1975). 4CL mRNA activity was determined by *in vitro* translation of total RNA using a rabbit reticulocyte lysate as described by Ebel et al. (1984) and a 4CL-specific antiserum (Ragg et al., 1981). Inhibition of 4CL enzyme activity in extracts of cell cultures by 4CL antiserum was analyzed by incubation for 0.5 h at 30°C and for 4 h at 4°C (Ragg et al., 1981) with appropriate amounts of antiserum, followed by treatment with 5 to 20 mg of protein A-Sepharose CL-4B (Pharmacia) for 1 h at 4°C.

RESULTS

Changes in 4CL Enzyme Activity and 4CL mRNA Activity and Amount in Elicitor-Treated Cell Cultures and Fungus-Challenged Roots

4CL total enzyme activity was stimulated 2- to 10-fold following treatment of suspension-cultured soybean cells

with a glucan elicitor fraction of *P. megasperma* f. sp. *glycinea*, with highest enzyme activity occurring between 8 and 12 h after the onset of treatment (Fig. 1). When differently substituted cinnamic acids are used as substrates in the enzyme assay, two isoforms of 4CL can be distinguished in soybean cells (Knobloch and Hahlbrock, 1975). In the experiment shown in Figure 1, the activity of isoform 1 of 4CL remained below the detection limits of the assay throughout the period analyzed, and, therefore, total enzyme activity represented mainly isoform 2. In a second experiment, it was confirmed (Hille et al., 1982) that the increase in enzyme activity was due mainly to an enhancement of the activity of isoform 2 of 4CL, whereas the activity of isoenzyme 1 remained almost unaffected. After 10 h of elicitor treatment, the activity of 4CL isoform 1 was $2.0 \mu\text{kat kg}^{-1}$ (untreated control $1.6 \mu\text{kat kg}^{-1}$) and that of isoform 2 was $8.4 \mu\text{kat kg}^{-1}$ (untreated control $3.1 \mu\text{kat kg}^{-1}$). Similar analyses with roots following infection with *P. megasperma* were not successful, very likely because of the lack of sensitivity of the 4CL enzyme assay used.

Changes in 4CL mRNA activity and amount were measured by using an antiserum to the enzyme from parsley (Ragg et al., 1981) and a cDNA (UV16) corresponding to parsley 4CL-1 (Lozoya et al., 1988). 4CL enzyme activity in extracts from soybean cell cultures was progressively inhibited following pretreatment with increasing concentrations of the heterologous antiserum (Fig. 2). The increase in 4CL enzyme activity in elicited cell cultures (Fig. 1) was preceded by an increase in 4CL mRNA activity and amount (Fig. 3, A and C). Highest levels of mRNA activity and amount were found 5 to 6 h after the onset of elicitor treatment (results not shown). Whereas the antiserum detected multiple *in vitro* synthesized proteins with a size of about 60 kD, the ^{32}P -labeled cDNA probe hybridized specifically to an mRNA with a size of 2 kb. An enhancement of 4CL mRNA activity

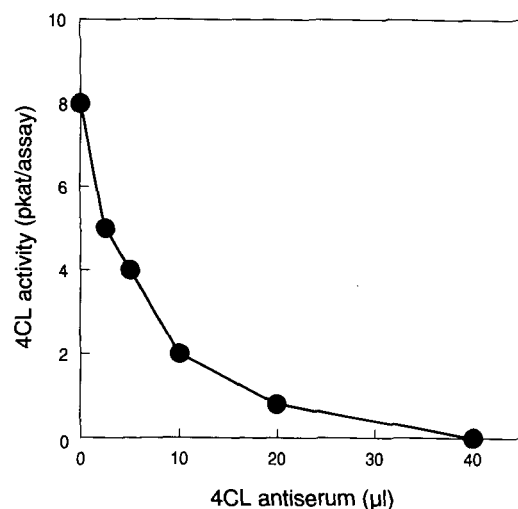


Figure 2. Effects of 4CL-specific antiserum on 4CL enzyme activity in soybean cell culture extracts. Inhibition of 4CL activity by the antiserum to the enzyme from parsley was analyzed by using increasing amounts of the antiserum, followed by treatment with protein A-Sepharose CL-4B.

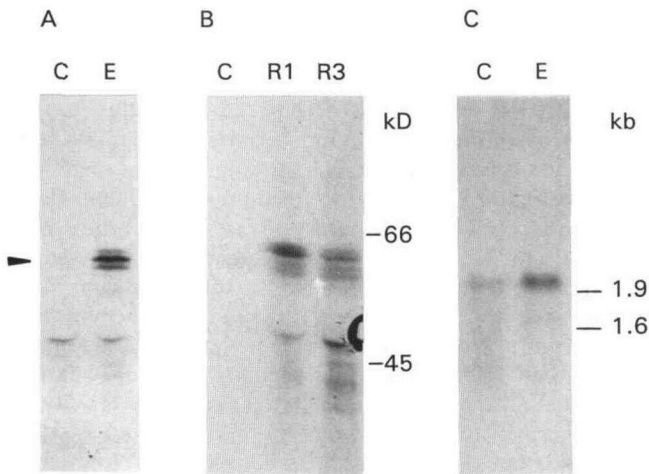


Figure 3. Changes in 4CL mRNA activity (A and B) and amount (C) in soybean cell cultures after treatment with β -glucan elicitors from *P. megasperma* (A and C) or in soybean roots after inoculation with zoospores of the fungus (B). Lane E, Elicitor-treated cells; lane R1, roots inoculated with race 1 of *P. megasperma*; lane R3, roots inoculated with race 3 of the fungus; lane C, water-treated control. Total RNA (A and B) and poly(A)⁺ RNA (C) were prepared after 5 h of elicitor treatment and 6 h of postinoculation. 4CL mRNA activity and amount were determined by *in vitro* translation using a rabbit reticulocyte lysate and a 4CL-specific antiserum to the enzyme from parsley and by northern blot analysis using a ³²P-labeled parsley 4CL cDNA, respectively. The protein size markers are shown in B, and the RNA size markers are shown in C; the arrowhead denotes proteins that correspond to the expected size of soybean 4CL.

was also observed in the incompatible (race 1) and compatible (race 3) interactions of soybean roots with *P. megasperma*, when measured 6 h postinoculation (Fig. 3B). It was again found that the antiserum cross-reacted with several *in vitro* synthesized proteins with a size of about 60 kD. The apparent complexity of the proteins detected by the 4CL antiserum hampered an unambiguous identification of 4CL in soybean. Therefore, we decided to use the parsley cDNA for the cloning procedure.

4CL-Specific cDNA Clones

A λ gt10 cDNA library was prepared from poly(A)⁺ RNA from elicitor-treated soybean cell-suspension cultures and screened with the parsley 4CL cDNA (UV16) (Lozoya et al., 1988). A first screen of approximately 5×10^4 recombinant plaque-forming units yielded four positive phages, which were purified through several cycles. The phages contained cDNA inserts with sizes of 1.0 to 1.5 kb. A second screen of approximately 9×10^4 plaque-forming units, using as a probe the soybean clone containing the largest insert (GM4CL16), yielded an additional four positive phages. None of the eight clones contained a full-size cDNA of the expected length of about 2 kb (Fig. 3C). The clones were analyzed by subcloning and sequencing. Two clones (GM4CL14 of 1.0 kb and GM4CL16 of 1.5 kb) were fully sequenced; the other six were analyzed from both the 5' and 3' ends for 300 to 600 bp.

The eight cDNAs were grouped in three classes on the basis of restriction maps and sequence analysis: (A) five independent clones (GM4CL1, GM4CL2, GM4CL8, GM4CL16, GM4CL31) that are distinguished by their length but show complete nucleotide homology; (B) two identical clones (GM4CL13, GM4CL32) that show several differences in the nucleotide and deduced amino acid sequences as compared to class A; (C) one clone (GM4CL14) that is distinguished from class A and B by larger differences (Fig. 4).

The results of DNA sequence analysis for typical clones in classes A, B, and C are summarized in Figure 4. An alignment of clones GM4CL13, GM4CL14, and GM4CL16 shows that clone GM4CL14 is distinguished from each of the other two clones by a larger degree of base exchanges in the protein-coding region, resulting in a higher level of amino acid differences. Many but not all of the differences may be considered to be conservative exchanges. The degree of homology at the nucleotide level is 65% between clones GM4CL16 and GM4CL14, 63% between GM4CL13 and GM4CL14, and 96% between GM4CL13 and GM4CL16. The largest cDNA (GM4CL16) contains an open reading frame of 423 amino acids. Southern blots revealed that under stringent conditions (0.1 \times SSC, 0.5% SDS, 60°C) no cross-hybridization between 0.7 kb *Eco*RI-*Kpn*I fragments (Fig. 4) of clone GM4CL14 and GM4CL16 occurred, whereas GM4CL16 hybridized to GM4CL13 under the same conditions (results not shown).

Alignment and comparison (Fig. 5, Table I) of the deduced amino acid sequences of GM4CL14 and GM4CL16 to those of previously isolated 4CL genes from parsley (Lozoya et al., 1988), potato (Becker-André et al., 1991), and rice (Zhao et al., 1990) reveal several characteristic features. A high degree of homology exists between GM4CL13 and GM4CL16 (97%), and there is less homology between GM4CL14 and GM4CL16 (71%). Intermediate degrees of similarity are found between the two soybean cDNAs, GM4CL14 and GM4CL16, and both PC4CL1 and ST4CL1 genes (68–80%). Less homology exists between the 4CL amino acid sequences of the three dicot plants and the monocot plant (ranging from 59–66%).

The data from sequence comparison suggest that the soybean cDNA clones indeed code for 4CL from this plant. This conclusion is further supported by the identification of extended similarity between deduced amino acid sequences of the presumed soybean 4CL cDNAs and those of the identified 4CL genes from parsley, potato, and rice. Of the several existing regions, two are marked in Figure 5. All of the 4CL proteins listed contain a highly conserved region very rich in Gly, Ser, and Thr that is followed by a conserved Lys (region I). It has been proposed (Bairoch, 1991) that region I designates a putative AMP-binding domain that is common to a number of prokaryotic and eukaryotic ATP-dependent enzymes. A second conserved sequence is that of region II, which contains one common Cys residue. The possibility has been discussed (Becker-André et al., 1991) that this Cys residue is associated with catalytic activity. Furthermore, the amino acid sequences of 4CL from plants other than soybean contain a total of six conserved Cys residues (Fig. 5). In the available sequences of soybean 4CL, all Cys residues are conserved.

GM4CL16 1 **GC**GAAGTTGATCATAAACACAGGCAATGTACGTGGACAAGCTCCGCAACCACGACGGCCGGAAGCTCGGCGAGGACTTCAAGGTCGTAACCGTCGACGATCCGCCGGAGAATGCCTCCAC
16 1 A K L I I T Q A M Y V D K L R N H D G A K L G E D F K V V T V D D P P E N C L H

GM4CL16 121 **TTCTCTGTCCTCTCGGAGGGCGAACGAGAGCGGACGTGCCAGAGGTGGAGATCCACCCGACGACGGGTGGCGATGCCCTTCTCCTCCGGCACGACGGGTTTACTAAAGGAGTGATTCTC**
16 41 F S V L S E A N E S D V P E V E I H P D D A V A M P F S S G T T G L P K G V I L

GM4CL16 241 **ACGCACAAGATTTAAACACGAGTGTGGCGCAGCAAGTTGACGGAGAGAACCCCTAACCTCTACCTCACCACCGGACGTCCTCTCGCTGCTTCCGCTCTTTACATATTCCTCGCTC**
16 81 T H K S L T T S V A Q Q V D G E N P N L Y L T T E D V L L C V L P L P H I F S L

GM4CL16 361 **AACAGTGTGCTATTGTGGCCCTCAGGGCGGGAGTGCAGTTTGTGTGATGCAGAAAGTTCGAGATCGGACACTGCTGGAGCTGATACAGCGCCACCGAGTGTCCGGTGGCGATGGTGGT**
GM4CL14 1 **GCCAAAGGTACCATTCTCTCATGCCAAAGTTCGATATTAACCTCTTGTCTCTCATTACAAAGCACAAAGTCACTATTGCCCTCTGCTCTC**
16 121 N S V L L C A L R A G S A V L L M Q K F E I G T L L E L I Q R H R V S V A M V V
14 1 > K A T I P D N S A H K K T I P

GM4CL16 481 **CCCCCGCTGGTGTGGCGTTGGCAAGAATCCGATGGTGGCGGATTTGACCTGAGTTCAAATACGGTTAGTGTCTCGGAGCTGCTCCCTTGGGGAAGGAGCTCGTGGAAAGCTCTCCGG**
GM4CL13 1 **GCTCCCTTGGGGAAGGAGCTCGAGGAGGCTCTCCGG**
GM4CL14 94 **CCTCCCAATGTTCTCGCCATTTCCAAATACCCGATCTCCCAAGTACGACCTGCTTCCATCAGAGTCTTGAAGTCCGGGGGAGCCCTCTGGGTAAAGAACTCGAAGACACTCTCAGA**
16 161 P P L V L A L A K N P M V A D F D L S S I R L V L S G A A P L G K E L E E A L R
13 1
14 32 I I S S D L H K Y V L K G D T

GM4CL16 601 **AACAGGGTGCCTCAAGCGTTTGGGACAGGGGTACGGATGACAGAAGCAGGACCAAGTGTCTTCCATGTCTTGGGCTTTGCAAAGCAACCTTTCCAAACAAAATCAGGCTCTTGTGGT**
GM4CL13 37 **AACAGGATGCTCAAGCTGTTTGGGACAGGGTTACGGGATGACAGAAGCAGGCGCCAGTGTCTCCATGTCTTGGGCTTTGCAAAGCAACCTTTCCAAACAAAATCAGGCTCTTGTGGT**
GM4CL14 214 **GCTAAATCCCCAAACGCCAACTTGGCCAGGATACGGAATGACTGAGGACAGGCTCTGTCTTAACTGTCTTGTAGCTTTTGTAAAGAACCGATAGACGTTAAACCAGGTGATGTTGGA**
16 201 N R M P Q A V L G Q G Y G M T E A G P V L S M C L G F A K Q P F Q T K S G S C G
13 13 V P
14 72 A K F N K T S A E I D V P A

GM4CL16 721 **ACCGTAGTCAGAAATGCAGAACTCAAGGTTGTGACCCCTGAAACTGGTCTCTCTTGGCTACAATCAACCCGGTGAATTTGCATCAGAGGGCAACAGATCATGAAAGGATATCTGAAC**
GM4CL13 157 **ACCGTAGTCAGAAATGCAGAACTCAGGTTGTGACCCCTGAAACTGGTCTCTCTTGGCTACAATCAACCCGGTGAATTTGCATCAGAGGGCAACAGATCATGAAAGGATATCTGAAC**
GM4CL14 334 **ACCGTTGTAAGAAATGCAGAGATGAAGAATTGTGATCCTGAAACCCGGTCACTCTTACCAGAAACCAATCCGGTGAATTTGCATAAGAGGGCAGCCAGATTATGAAAGGTTATCTAAAT**
16 241 T V V R N A E L K V V D P E T G R S L G Y N Q P G E I C I R G Q Q I M K G Y L N
13 53 R
14 112 M I H P R S D

GM4CL16 841 **GATGAGGCAGCGACAGCATCGACCATAGATTAGAGGGTTGGCTTCACACCGGTGATGTTGGCTACGTAGATGATGATGACGAAATTTTCATTTGTCAGGGTGAAGAACTCATCAA**
GM4CL13 277 **GATGAGAAAGCGACAGCATTGACCATAGATTAGAGGGTTGGCTTCACACCGGTGATGTTGGCTATGTAGATGAAGATGATGAAATTTTCATTTGTCAGGGTGAAGAACTCATCAA**
GM4CL14 454 **GATGAGAGGGCTACAGAGAAACCATAGACAAAGATGGTGGTGCACACAGGTGATCAGGTTACATCGACGATGACGATGAGTATTTCATCGTTGACAGGCTCAAGGAATGATCAA**
16 281 D E A A T A S T I D S E G W L H T G D V G Y V D D D D E I F I V D R V K E L I K
13 93 K L E
14 152 G R E R K D I I L L

GM4CL16 961 **TATAAAGGCTTCCAGGTGCCCCCTGCAGAACTTGAAGGCTTCTTGTAAAGCCATCCCTCCATTGCAGATGCAGCTGTTGCCCAAAAAGGATGTTGCTGCTGGTGAAGTTCCTGTGTCC**
GM4CL13 397 **TATAAAGGCTTCCAGGTGCCCCCTGCAGAACTTGAAGGCTTCTTGTAAAGCCATCCCTCCATTGCAGACGCGAGCTGTAGTCCCAAAAAGGATGTTGCTGCTGGTGAAGTTCCTGTGTCC**
GM4CL14 574 **TACAAGGATTTCAAGTGGCTCCAGCTGAACTTGAAGCCCTTCTTCACTCACTCAAGATCTCTGATGCTGCTGTTGTTCCAAATGAAGGATGAAGCCCGGGAGAGTACTGTGTGA**
16 321 Y K G F Q V P P A B L E G L L V S H P S I A D A A V V P Q K D V A A G E V P V A
13 133
14 192 A A L T K S M E

GM4CL16 1081 **TTCTGTTGAGATCAACCGC---TTTGTACTAAGTGAAGAGGCTGTAAAGAGTPTATAGCTAAACAGGTAGTGTTTTACAAAAGACTGCACAAAGTTTATTTTGTTCATGCTATTC**
GM4CL13 517 **TTCTGTTGAGATCAACCGC---TTTGTACTAAGTGAAGAGGCTGTAAAGAGTPTATAGCTAAACAGGTAGTGTTTTACAAAAGACTGCACAAAGTTTATTTTGTACATGCTATTC**
GM4CL14 694 **TTTGTGTCATATCAATGGTTATACCGACACACCCGAGGATGAAATTAAGCAGTTTATCTCCAAACAGGTGGTGTTTTACAAAAGATAAACCGAGTATTCTTCATTGATGCAATTC**
16 361 F V V R S N G - P D L T E E A V K E F I A K Q V V F Y K R L H K V Y F V H A I P
13 173
14 232 I Y T T D E I Q S I N R F I D

GM4CL16 1198 **AAGTCTCCATCAGGAAAGATATTAAAGAAAGACCTCAGAGCAAAGCTAGAAACCGCCGCACTCAGACGCCTTAAAGGCTAGCTAGAACCTGCCCCCTTTTCTTGGCAATAT-TTCC**
GM4CL13 634 **AAGTCTCCATCAGGAAAGATATTAAAGAAAGACCTCAGAGCAAAGCTAGAAACCGCCGCACTCAGACGCCTTAAAGG---GCTAGAACCTG-CCCCCTTTTGGCAATATCTTTCT**
GM4CL14 814 **AAGTCAACCTCAGGCAAAATCTTGGCAAGGATCTAAGAGCAAAGATAGCGGCAAGTGTCCAAAATGAAGTAAACCAAACCCATCATGTTATGGTGTATTATACGATGCCCGCATAA**
16 400 K S P S G K I L R K D L R A K L E T A A T Q T P
13 212
14 272 I A A S V P K

GM4CL16 1317 **TTATTTTTTATTATATTATGGTTTCAACAGTGAATTTACGTATCAATGCTTCACGGCATTAAAGCCAA--TCCGAGAAGCAGTTGCACTTACGTATATCATTTGATTTTTATGTCCA**
GM4CL13 750 **TTTTTATTATTATATTATGGTTTCTCAGTGTAAATTTGGTATCAATGCTTCACTGCAATTAAGTCAAAGTCCGAGAAGCAGTTGCACTTACACATAATCATTTGATTTTTTGTGTCCA**
GM4CL14 934 **GCAAGCTTCAACAAAAGGAAGAGCGCTGTGCTGTATCCCGTAGCTTTTACGTATTTTCTCTTACGTAAACCGGTGTATAAGTTACAGTGTGATTTTCTTAC**

GM4CL16 1435 **TTGTCCACCACCATGCTGTATTGTGGTTTTAGATCACTAAATTTTCGAGACCTTGTAA**
GM4CL13 870 **TTGCCACCACCATGCCTGTATTGTGATTTTAG**

Figure 4. DNA sequence of 4CL cDNA clones GM4CL13, GM4CL14, and GM4CL16 and deduced amino acid sequences of these clones. For clones GM4CL13 and GM4CL14, only those amino acids are shown that differ from clone GM4CL16. The DNA line also indicates the start points (>) of the other class A (numbers 1, 2, and 8) or class B (number 32) clones and the *EcoRI-KpnI* fragments of clones GM4CL14 and GM4CL16, respectively, used as gene-specific probes (boldface letters).

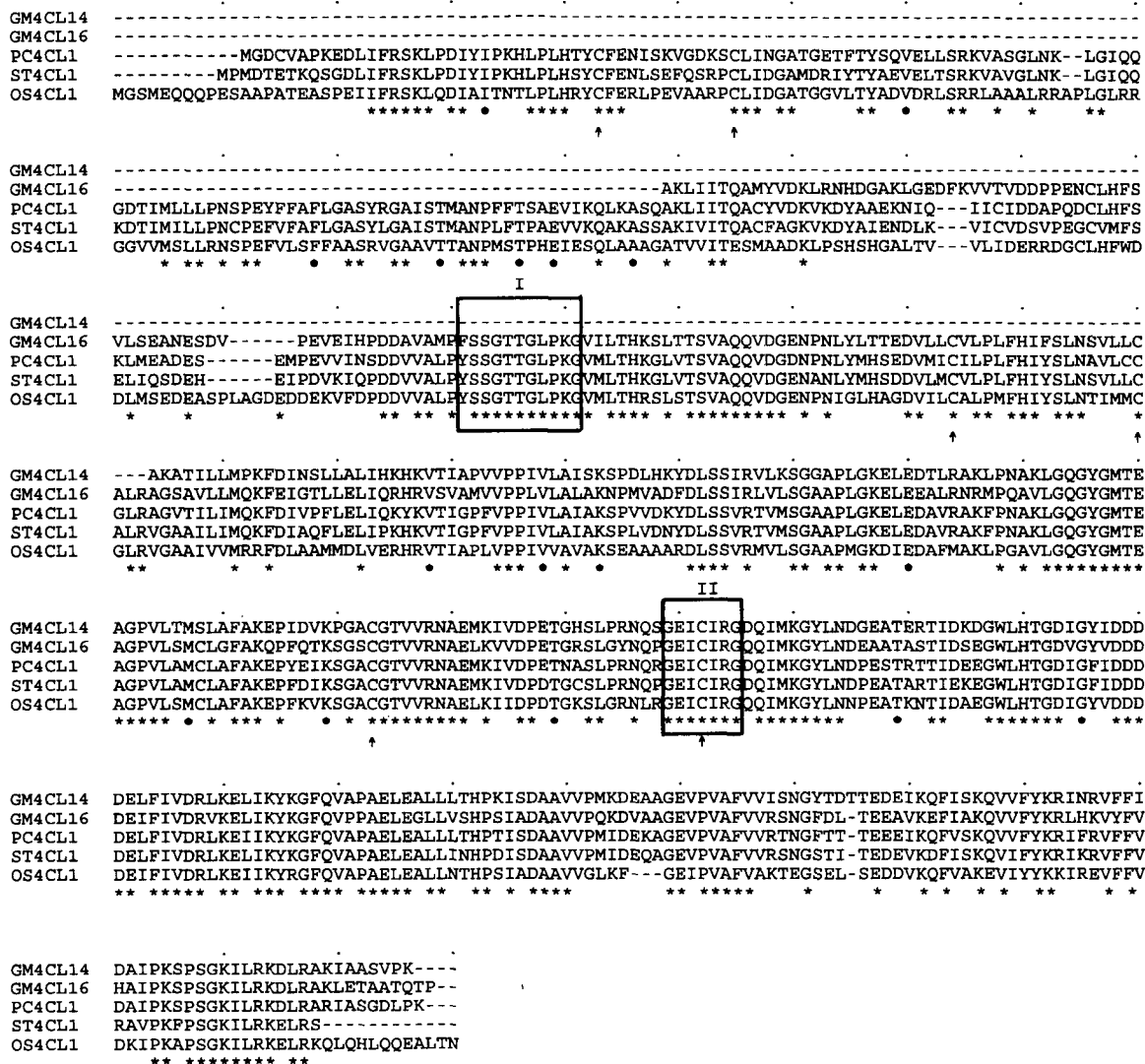


Figure 5. Comparison of the deduced amino acid sequences of soybean (GM4CL14 and GM4CL16), parsley (PC4CL1), potato (ST4CL1), and rice (OS4CL1) 4CL. Symbols denote amino acid identity (*) and conserved Cys residues (†); boxes I and II show regions of extended sequence identity suspected to be involved in enzyme catalysis (Bairoch, 1991; Becker-André et al., 1991). Sequence comparison was performed using the program CLUSTAL on a Genius computer (DKFZ, Heidelberg, Germany).

Table I. Relationships (percentage of identity at the protein level) between soybean 4CL and 4CL from other plants

Sequence compilation and evaluation was performed using the ALIGN2 program of M. Trippel (Infomed, Freiburg, Germany). GM, *G. max*; OS, *O. sativa*; PC, *P. crispum*; ST, *S. tuberosum*. Values in parentheses are the percentages of identity at the nucleotide level

	GM4CL13	GM4CL14	GM4CL16	PC4CL1	ST4CL1	OS4CL1
GM4CL13	100 (100)	69 (63)	97 (96)	70	70	66
GM4CL14		100 (100)	71 (65)	80	80	64
GM4CL16			100 (100)	68	69	61
PC4CL1				100	79	59
ST4CL1					100	61

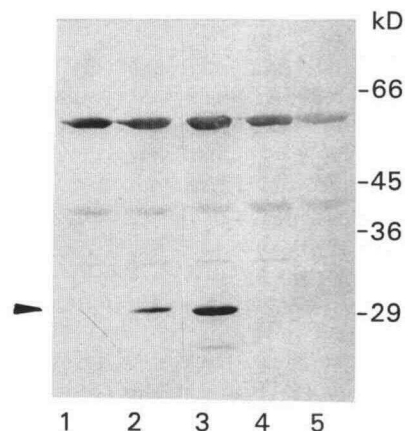


Figure 6. Western blot analysis of recombinant 4CL protein after expression of GM4CL13 cloned in pTZ19R in *E. coli* strain JM109. Lane 1, pTZ19R in *E. coli* strain JM109; lane 2, correct orientation of cDNA, uninduced control; lane 3, correct orientation of cDNA, induced with IPTG; lane 4, opposite orientation of cDNA, uninduced; lane 5, opposite orientation of cDNA, induced with IPTG. The arrowhead denotes the protein of about 30 kD that specifically cross-reacted with 4CL antiserum. Protein size markers are shown in kD.

To provide further evidence for the identity of the soybean 4CL clones, GM4CL13 was inserted in the correct phase into the coding region of β -galactosidase in vector pTZ19R and expressed in *E. coli*. The bacterial extracts were analyzed for cross-reacting protein using the 4CL-specific antiserum to the enzyme from parsley. Western blots demonstrated (Fig. 6) the IPTG-stimulated production of a cross-reacting fusion protein of the expected size of about 30 kD. No protein was detected in extracts from *E. coli* cells transformed either with the vector alone or with vector containing GM4CL13 in the opposite orientation.

Genomic Organization of Soybean 4CL

Genomic DNA from soybean cell cultures was digested with a variety of restriction endonucleases and analyzed by

Southern blot hybridization (Fig. 7). When *EcoRI-KpnI* fragments of GM4CL14 and GM4CL16, making up 0.7 kb (Fig. 4), were used as probes, each revealed two or more bands with *EcoRI*- or *HindIII*-digested genomic DNA. The *BamHI*-cleaved DNA showed one band with GM4CL14 and three bands of comparable intensity with GM4CL16. Similar results were obtained with genomic DNA from soybean leaves and hypocotyls. The results showed that each 4CL cDNA probe hybridized to a distinct set of bands, indicating that 4CL is encoded by a small gene family in soybean, although the possibility of allelic variants cannot be ruled out. GM4CL14 and GM4CL16 very likely represent members of two different sets of genes, with GM4CL13 being allelic to GM4CL16.

Changes in 4CL Transcript Levels in Soybean in Response to Elicitor Treatment and to Infection

The *EcoRI-KpnI* fragments of GM4CL14 and GM4CL16 were used as hybridization probes, and RNA samples from elicitor-treated soybean cell-suspension cultures and from *P. megasperma*-infected soybean roots were analyzed for induced changes in 4CL mRNA amounts. The duration of treatment was 5 h for cell cultures and 6 h for roots, which under identical conditions was shown previously to cause large coordinate enhancements in the mRNA levels of two enzymes of phytoalexin biosynthesis, Phe ammonia-lyase, and CHS (Habereder et al., 1989).

The results obtained with elicitor-treated cell cultures are shown in Figure 8. Both 32 P-labeled cDNA probes specifically hybridized to transcripts of a size of approximately 2.0 kb on gels containing total RNA. A fraction of the transcripts detected by using GM4CL14 was present in nonelicited control cell cultures. The level of the GM4CL14-specific transcripts was only slightly affected by elicitor treatment, the average enhancement being about 1.5-fold when the results from three independent experiments were compared (Fig. 8A). The transcript level in the control cultures at 5 h was very similar to that at 0 h (results not shown). A different situation was encountered with a second fraction of transcripts detected upon hybridization using the GM4CL16 probe (Fig. 8B). Apparently no mRNA from nonelicited control cell cultures hybridized to GM4CL16, but the mRNA level was markedly

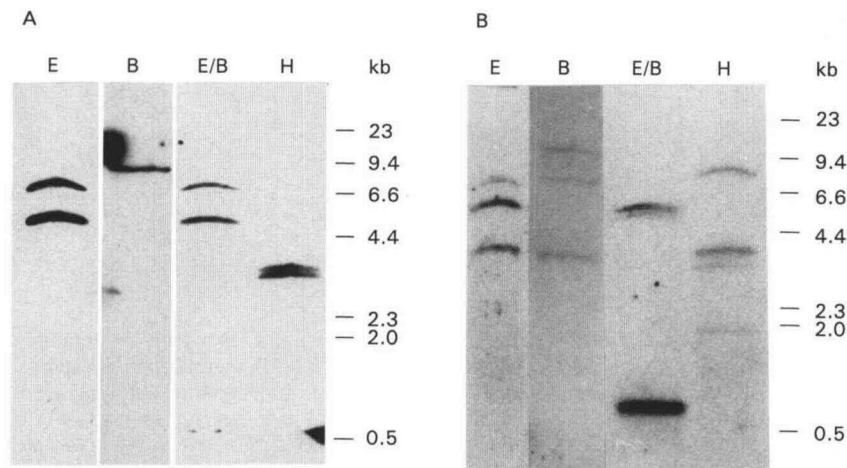


Figure 7. Southern blot analysis of soybean genomic DNA. DNA samples (10 μ g) were digested with the restriction endonucleases *EcoRI* (E), *BamHI* (B), *HindIII* (H), or a combination of the enzymes (E/B), electrophoresed on 0.8% agarose gels and blotted onto nitrocellulose membranes. A, Membrane analyzed with the 32 P-labeled *EcoRI-KpnI* fragment of GM4CL14; B, membrane analyzed with the 32 P-labeled *EcoRI-KpnI* fragment of GM4CL16. DNA size markers are shown in kb.

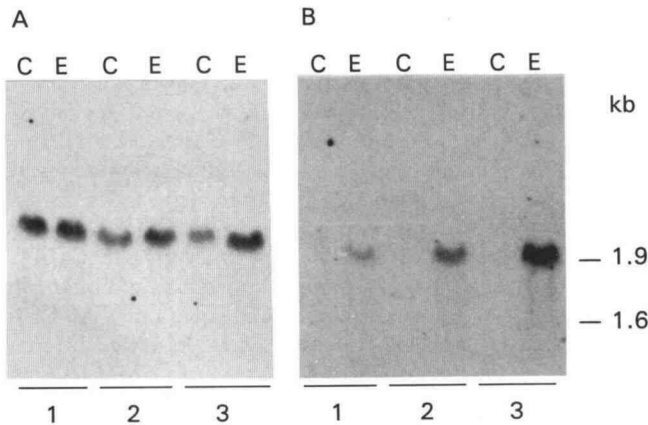


Figure 8. Changes in 4CL mRNA amount in soybean cell cultures after treatment with *P. megasperma* β -glucan elicitors for 5 h. Total RNA (20 μ g) was denatured and fractionated on 1.2% agarose gels containing formaldehyde and transferred to nitrocellulose membranes. A, Blot probed with the 32 P-labeled *EcoRI-KpnI* fragment of GM4CL14; B, blot probed with the 32 P-labeled *EcoRI-KpnI* fragment of GM4CL16. Lane C, Untreated control cells; lane E, elicitor-treated cells. The results from three independent experiments are shown (lanes 1–3). RNA size markers are shown in kb.

enhanced after 5 h of elicitor treatment. The degree of enhancement of the mRNA level was somewhat variable in the three experiments reported in Figure 8, a variation that was usually also observed in elicitor stimulation of 4CL enzyme activity in different experiments (see above). Again, there was no difference in the transcript levels of control cell cultures at 0 and 5 h. Because GM4CL16 and GM4CL13 cross-hybridized under stringent conditions, the band detected in Figure 8B could consist of a mixture of transcripts that have some structural differences but are of very similar size.

An analysis similar to that shown for elicitor-challenged cell cultures was performed with roots of 2-d-old soybean seedlings following infection with zoospores of *P. megasperma* (Fig. 9). Total RNAs extracted from roots after infection with race 1 of the fungus (incompatible interaction, plant resistant) and from roots after infection with race 3 (compatible interaction, plant susceptible) were compared with RNA from water-treated control roots. As with cell cultures, GM4CL14-specific mRNA of approximately 2.0 kb was detected in control roots, and its level was enhanced about 2-fold in the incompatible interaction and was only slightly affected in the compatible interaction (Fig. 9A). Very low levels of GM4CL16-specific transcripts were present in control roots and in roots during the compatible interaction, whereas the level of these transcripts was strongly enhanced in roots during the incompatible interaction (Fig. 9B). The large differential effects on the levels seen for the GM4CL16-specific transcripts resembled those found for changes in CHS mRNA levels (Fig. 9C) when analyzed with a gene-nonspecific probe of bean (pCHS1, Ryder et al., 1984). We conclude from these results that GM4CL14 and GM4CL16 represent gene (class)-specific cDNAs that can be utilized to reveal differential regulation of 4CL genes of soybean in response to elicitor challenge and pathogen attack.

DISCUSSION

We have isolated several cDNA clones that very likely encode at least three 4CL isoenzymes in soybean. The identification of the 4CL cDNAs from soybean is based on cross-hybridization with a well-characterized 4CL cDNA from parsley, on sequence similarities with several 4CL genes of known structure, on the detection, using 4CL antibodies against the parsley enzyme, of cross-reacting material after *in vitro* expression of a putative 4CL cDNA clone, and on the size and the regulation of the cross-hybridizing mRNA.

The cDNA clones have been grouped into three classes based on sequence analysis, representative examples for each of the classes being GM4CL13, GM4CL14, and GM4CL16. Alignment and sequence comparison at the nucleotide and deduced amino acid level predicted structural differences at the gene, mRNA, and protein levels, in agreement with the earlier distinction between two 4CL isoenzymes based on protein isolation and substrate specificity (Knobloch and Hahlbrock, 1975). Between the soybean 4CL clones GM4CL14 and GM4CL16, the nucleotide and amino acid sequences are only 65 and 71% similar, respectively. More closely related are clones GM4CL13 and GM4CL16 (96 and 97%). Nucleotide sequence differences, thus, allowed 4CL cDNAs GM4CL14 and GM4CL16 to be used to generate probes for gene class-specific analysis.

Genomic blots with soybean DNA using the two soybean 4CL cDNAs as probes indicate the presence of either more than two genes or possible allelic variants of a few genes for 4CL in this plant. Both the structure of the cDNA clones and the genomic organization of 4CL, therefore, demonstrate that GM4CL14 and GM4CL16 very likely represent members of two different classes of genes. It appears likely that GM4CL14 and GM4CL16 are correlated with the two known isoenzymes of soybean, but it cannot be excluded that the number of isoforms might be even larger than deduced from previous work at the protein level (Knobloch and Hahlbrock, 1975). The complexity of 4CL in soybean at the gene and protein level is quite different from that of 4CL from other plants (Lozoya et al., 1988; Zhao et al., 1990; Becker-André et al.,

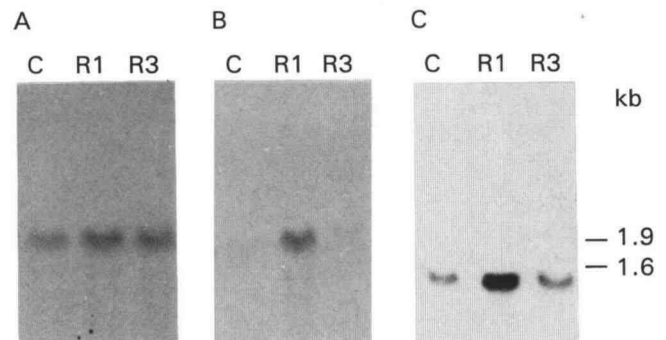


Figure 9. Changes in 4CL (A and B) and CHS (C) mRNA amount in soybean roots inoculated for 6 h with *P. megasperma* zoospores. The analysis was done as described for Figure 6. The blots were probed with 32 P-labeled *EcoRI-KpnI* fragments of GM4CL14 (A) or GM4CL16 (B) or 32 P-labeled CHS cDNA (C). RNA size markers are shown in kb.

1991). In both parsley (Douglas et al., 1987; Lozoya et al., 1988) and potato (Becker-André et al., 1991), two closely related, structurally similar 4CL genes exist that have only a few nucleotide differences in the coding region, which result in three (parsley) or four (potato) amino acid differences in 4CL isoenzymes.

In contrast to the overall differences there are several structural relationships between 4CL in soybean, parsley, and potato. Antibodies raised against parsley 4CL immunoprecipitated several proteins of about 60 kD after in vitro translation of mRNA from soybean cell cultures and one fusion protein of the expected size in extracts from *E. coli* cells transformed with GM4CL13. These results indicate that one or more immunogenic epitopes of 4CL from different plants are similar. This notion is supported by the identification of several separate regions of extended amino acid sequence identity among soybean, parsley, potato, and rice 4CL. It has been pointed out (Bairoch, 1991) that a number of prokaryotic and eukaryotic enzymes, including 4CL, which all probably act via an ATP-dependent covalent binding of AMP to their substrate, share a region of striking sequence similarity (region I in Fig. 5). This conclusion can now be extended to soybean 4CL. The possible functional role of a conserved Cys residue in region II (Fig. 5) in the intermediate, covalent binding of the activated substrate (Lipmann, 1980) has yet to be determined for plant 4CL (Becker-André et al., 1991). On the other hand, distinct amino acid differences might be responsible for the different abilities of the soybean 4CL isoenzymes to utilize 4-coumarate, caffeate, ferulate, and sinapate as substrates (Knobloch and Hahlbrock, 1975).

Induction of phytoalexin synthesis in soybean following fungus infection or elicitor treatment involves a set of enzymes of the biosynthetic pathway (Ebel and Grisebach, 1988). Among these, 4CL has been proposed to play an important role at a branch point in phenylpropanoid metabolism by regulating the flux of substituted cinnamate derivatives into phytoalexin production as well as into the formation of other inducible or constitutive phenylpropanoid compounds. One model for explaining the demand for differently substituted cinnamate CoA esters in the several branches of phenylpropanoid metabolism is the differential regulation of 4CL genes leading to the expression of isoenzymes that exhibit different substrate specificities. The cDNA fragments of GM4CL14 and GM4CL16 generated during these studies, which did not cross-hybridize, promised to be useful for studies of the differential transcription of 4CL genes.

Our results show that gene (class)-specific 4CL mRNAs and isoenzyme activity levels are differentially regulated in response to both pathogen attack of soybean roots and elicitor treatment of soybean cell cultures. On the one hand, the activity level of 4CL isoenzyme 2 and the level of GM4CL16-specific mRNA are rapidly and highly enhanced when treated with elicitor; on the other hand, 4CL isoenzyme 1 activity and the level of GM4CL14-specific mRNA are only slightly affected. Despite this obvious correlation between changes of isoenzyme activities and mRNA amounts, the assignment of the isolated 4CL cDNAs to isoenzyme 1 and 2 in soybean cannot be done until representative full-size cDNAs are isolated and expressed in vitro (Lozoya et al., 1988).

The analysis of soybean roots infected with *P. megasperma* again revealed characteristic differences in induction of gene (class)-specific 4CL mRNAs between the incompatible and compatible host-pathogen interaction. The observed pattern resembles that found for Phe ammonia-lyase and CHS mRNA in this system (Habereeder et al., 1989). It is interesting that GM4CL14 and GM4CL16 also appear to be regulated in an organ-specific manner in this plant (S. Reinold and J. Ebel, unpublished results). These 4CL expression patterns are quite different from those in other plants. Neither in parsley nor in potato has any differential regulation of 4CL genes yet been demonstrated in response to pathogen attack (Hahlbrock and Scheel, 1989; Becker-André et al., 1991).

The existence of 4CL isoenzymes in soybean possessing distinct substrate specificities and showing differential regulation of expression supports the model for a role of 4CL in metabolic channeling in phenylpropanoid branch pathways in this plant. Further analyses of the promoter structures of selected genes may contribute to an understanding of the regulation of 4CL genes in soybean. They may demonstrate the presence of specific regulatory elements that are related to the various functions of 4CL during plant development and in response to environmental stimuli (Hahlbrock and Scheel, 1989; Ebel and Scheel, 1992).

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