Identification and Genetic Regulation of the Chalcone Synthase Multigene Family in Pea

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Chalcone synthase (CHS) is a key enzyme in the biosynthesis of diverse flavonoids involved in disease resistance, nodulation, and pigmentation in pea. We describe a multigene family encoding CHS and the effects of two regulatory loci, \(a\) and \(a2\), on the pattern of expression of three of its member genes. Two of the genes, CHS1 and CHS3, are expressed in both petal and root tissue, whereas expression of a third gene, CHS2, is detected only in roots. The products encoded by the \(a\) and \(a2\) loci are required for the expression of the CHS1 gene and for wild-type levels of expression of the CHS3 gene in petal tissue. In root tissue, all three CHS genes are expressed and induced by \(\text{CuCl}_2\) regardless of the genotype at the \(a\) and \(a2\) loci. These results show that the various members of the CHS multigene family interact in diverse ways with multiple genetic signals in the plant, providing a basis for the differential expression of these genes. Spatially specific genetic regulation of distinct members of a multigene family has been clearly demonstrated.

INTRODUCTION

A common feature of multigene families is that their members are expressed in different ways in response to environmental and developmental signals. There have been very few studies on how the pattern of expression of these genes is genetically controlled. The multigene family chalcone synthase (CHS), an enzyme required for flavonoid biosynthesis, is a good example of a gene family that is differentially expressed in plants and for which potential regulatory mutants are available. Here we describe the regulation of the CHS multigene family in pea. This species has well-characterized genetics and also has the advantage that the role of various flavonoids has been extensively studied.

Chalcone synthase catalyzes the first step in phenylpropanoid biosynthesis specific to the formation of flavonoid pigments and isoflavonoids. Three acetate residues from malonyl-CoA are condensed with one molecule of 4-coumaroyl-CoA to yield naringenin-chalcone (Kreuzaler and Hahlbrock, 1975; Heller and Hahlbrock, 1980). Isomerization and further substitution of this central intermediate lead to the synthesis of flavonols, flavones, flavonoids, anthocyanins, and isoflavonoids (Hahlbrock, 1981; Dixon, Dey, and Lamb, 1983). Three different types of flavonoids have been extensively documented in pea. Pisatin, an isoflavonoid, has been shown to function as a phytoalexin because the ability of Neotria haematococca to demethylate and tolerate pisatin from epicotyls of pea seedlings is associated with its virulence (Tegtmeier and van Etten, 1982). Specific types of flavones and flavanones have been identified in pea roots as signals for activation of nodulation genes in Rhizobium (Firmin et al., 1986; Peters, Frost, and Long, 1986; Redmond et al., 1986). Another class of flavonoids, anthocyanins, is responsible for pigmentation in pea flowers, stipules, stems, axil rings, pods, and testas, and several loci altering pigmentation have been identified (Statham, Crowden, and Harborne, 1972). Of particular interest are the \(a\) and \(a2\) loci, which are essential for pigment biosynthesis (Mendel 1866; Marx, Weedon, and Muehlbauer, 1989).

CHS has been shown to be encoded by a multigene family in bean (Ryder et al., 1987) and petunia (violet 30) (Koes et al., 1987). Environmental stress such as UV light (Kreuzaler et al., 1983), phytopathogens and elicitors (Ryder et al., 1984; Bell et al., 1986; Ryder et al., 1987), or wounding (Bell et al., 1986; Ryder et al., 1987) leads to the differential expression of the multigene family encoding CHS in bean. In petunia, the expression of different CHS members was found to be flower specific, light dependent, and developmentally controlled; in addition, the expression of CHS genes can be induced in young seedlings and cell suspension cultures by illumination with UV light (Koes, Spelt, and Moi, 1989).

Here we describe how two regulatory loci, \(a\) and \(a2\), affect the pattern of expression of different members of the CHS multigene family in pea. Three members of the family were identified and their expression was analyzed in petals and root tissue that had been treated with the abiotic elicitor \(\text{CuCl}_2\). The expression of these members of
the multigene family is regulated in different ways by both
the a and a2 loci. In flower tissue, CHS1 gene expression
is absent in a and a2 mutants. CHS3 gene expression is
also absent in a2 mutants but is quantitatively reduced in
a mutants. All three classes of CHS genes are expressed
in root tissue regardless of the genotype of the a and a2
loci. These results show that the various members of the
CHS multigene family interact in diverse ways with multiple
genetic signals in the plant, providing a basis for the
differential expression of these genes.

RESULTS

Isolation of Chalcone Synthase cDNAs

Poly A+ RNA isolated from purple-violet flowers was used
to prepare a cDNA library in pUC19. When this was
screened at low stringency with the heterologous bean
CHS probe, several positive clones were detected. One of
these, pCHS1, had an insert of 1.47 kb that showed strong
nucleotide sequence homology with the CHS cDNA iso-
lated from bean (78%) (Ryder et al., 1984), and the de-
duced amino acid sequence showed good homology with
the pea genome. The hybridization of all three probes to
the pea genome not accounted for by the hybridization of
pCHS1 and pCHS2 at high stringency. There is some
hybridization of pCHS2 to a fragment of the same size as
that detected at high stringency with pCHS1, but it is not
clear whether this is due to cross-hybridization to the same
fragment or to a different fragment of similar size. Copy
number reconstruction indicated that the genomic frag-
dments detected using pCHS1 was present at single copy in
the pea genome. The hybridization of all three probes to
multiple genomic fragments at low stringency and the
subsequent loss of hybridization signal to some of these
fragments at high stringency suggests that CHS in pea is
encoded by a multigene family, as in bean (Ryder et al.,
1987) and petunia (Koes et al., 1987). At low stringency,
the different members of the multigene family are detected,
whereas at high stringency a subclass of the CHS multi-
genome specific for the cDNA used as a probe is seen.
The single band detected by CHS1 at high stringency
preumably corresponds to the gene encoding CHS1
mRNA, whereas the multiple bands seen at high stringency
with pCHS2 probably include the gene encoding CHS2

Chalcone Synthase Is Encoded by a Multigene Family
in Pea

When the two pea CHS cDNAs were used to probe EcoRl-
digested DNA under low-stringency conditions, the hybrid-
ization pattern was very similar to that seen with the CHS
cDNA isolated from bean, with the majority of the genomic
DNA fragments detected being common to all three
probes, as shown in Figure 2. At high stringency, there
was little cross-hybridization between the CHS probes and
none detectable between the bean CHS probe and pea
dNA. A single DNA fragment was detected using pCHS1
as a probe, whereas pCHS2 formed hybrids with several DNA
fragments ranging in size from 2.3 kb to 11.7 kb
(Figure 2). Some of the EcoRl fragments that hybridized
to the pCHS2 probe at low stringency failed to do so at
high stringency, and others showed disproportionate loss
of hybridization signal at the higher temperature. This
suggests that there are further CHS DNA sequences in
the pea genome not accounted for by the hybridization of
pCHS1 and pCHS2 at high stringency. There is some
hybridization of pCHS2 to a fragment of the same size as
that detected at high stringency with pCHS1, but it is not
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Figure 1. Alignment of the Deduced CHS Amino Acid Sequence
of Pea with That of Other Species.

The consensus sequence was derived from eight different plant
species (Niesbach-Kløsgen et al., 1987). The bean CHS sequence
is from Ryder et al. (1984). The dots indicate those amino acids
that are common among the three CHS shown but not the
consensus.
B. J. I. W. M. -9.42 -6.55 -5.15 -4.36 -3.53 -2.32

Figure 2. Genomic DNA Gel Blot Analysis of Jl 336 (Wild Type) Pea DNA.

DNA (5 μg) was digested with EcoRI and size fractionated in a 0.8% agarose gel. After blotting, the strips were hybridized with the probes indicated below and were either washed at low stringency (LS) (2 x SSC, 0.05% SDS, 65°C) or at high stringency (HS) (0.1 x SSC, 0.05% SDS, 65°C). The fragment indicated with a dot in (B) could be the same one as that detected at HS with pCHS1 in (C).

(A) Hybridized with a CHS cDNA isolated from bean (Ryder et al., 1984).
(B) Hybridized with pCHS2.
(C) Hybridized with pCHS1.

mRNA, in addition to other closely related members of the multigene family. Restriction fragment length polymorphism analysis of an F2 population segregating for A and a using pCHS1 and pCHS2 as probes demonstrated that at least some of the genomic fragments detected by pCHS2 are at a different locus from that detected by pCHS1 (data not shown). Hence, these two members of the pea CHS multigene family are not clustered in the pea genome as are some of the CHS genes of the bean multigene family (Ryder et al., 1987).

**CHS1 Expression in Petal Tissue but not Roots Is Affected by Both the a and a2 Loci**

The most obvious phenotype of a plant carrying a mutation at either the a or a2 locus is an absence of pigmentation in flowers, as shown in Figure 3, and anthocyanin pigment is absent throughout the plant. The tissue specificity of CHS1 expression, and the effect the a or a2 loci have on this expression, was determined by S1 analysis. A uniformly labeled 5’ fragment of pCHS1 was used to probe total RNA isolated from petal tissue, uninduced, and CuCl2-induced root tissue of a2 mutants, a mutants, and the wild type. This probe contained a stretch of vector sequence to ensure that DNA:DNA hybrids could be distinguished from DNA:RNA hybrids after gel electrophoresis. In petal tissue, a fragment corresponding to the full-length protected fragment was detected only in wild type, as seen in Figure 4. Restriction of CHS1 gene expression to the wild type may have been due to differences in genetic background because the lines used were not isogenic. To overcome this difficulty, RNA was extracted from purple-violet or white petals from eight plants segregating at the a and a2 loci. This experiment gave the same result as before, i.e., CHS1 gene expression only occurred in purple-violet petal tissue, confirming that both the a and a2 loci affect the expression of CHS1 in petal tissue (Figure 4, I and II). An S1-protected fragment of the same size was also detected in root tissue isolated from all three lines, the intensity of which increased 12 hr after induction with CuCl2. The a2 mutant contained much less of this protected fragment than the other genotypes (Figure 4). However, because these lines were not isogenic, it is not clear whether this quantitative variation is due to the a2 locus or to differences in the genetic backgrounds. These data indicate that in roots, the a locus and probably the a2 locus have no effect on the CuCl2 inducibility of CHS1, although the a2 locus may have a quantitative effect on its expression.

Figure 3. Phenotypes of Wild-Type (w-type), a Mutant (a/a), and a2 Mutant (a2/a2) Flowers.
The a and a2 Loci Regulate CHS1 Expression in \textit{trans}

These data do not allow us to differentiate between a regulatory or a structural role for the a and a2 loci. The allele of either of these loci that confers the white-flowered phenotype may correspond to a mutation in the promoter region of the CHS1 structural gene that would prevent its expression in flower tissue, but would have no effect on its CuCl2-inducible expression in root tissue; alternatively, either or both could represent regulatory genes for CHS.

To distinguish between these possibilities, a restriction fragment length polymorphism analysis of \( F_2 \) populations segregating for the A and a or A2 and a2 alleles was carried out. Genomic DNA was isolated from the leaves of these \( F_2 \) populations, digested either with EcoRI (for the \( F_2 \) population segregating for A and a) or BamHI (for the \( F_2 \) population segregating for A2 and a2), and, after electrophoresis, DNA gel blotted and probed with pCHS1. As described previously, under conditions of high stringency, a single genomic EcoRI DNA fragment hybridized to this probe in DNA isolated from the wild type, a, and a2 mutants.

Analysis of the first \( F_2 \) population is presented in Figure 5A and demonstrates that the EcoRI DNA fragment detected in a mutants was approximately 12 kb and that in wild type was 8.3 kb in length. If these fragments corresponded to the a locus, then only the white-flowered plants

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DNA (5 \( \mu \)g) isolated from leaf tissue of purple-violet (P)- and white (W)-flowered plants of an \( F_2 \) population segregating for A/a or A2/a2 was digested with EcoRI or BamHI and fractionated in a 0.8% agarose gel. Both panels were probed with pCHS1 and washed at high stringency.

(A) EcoRI digests of DNA isolated from an \( F_2 \) population segregating for A/a.

(B) BamHI digests of DNA isolated from an \( F_2 \) population segregating for A2/a2.
from purple-violet petals, as shown in Figure 6, demonstrating that the presence of CHS protein segregates with the dominant allele at the a locus, although the gene encoding CHS1 does not. Our results contradict those of Hrazdina and Weedon (1986), who claimed that the a mutation did not affect the quantity of CHS protein. However, the protein they detected had a much larger molecular weight than their control CHS, and so it is unlikely that it corresponded to the chalcone synthase enzyme. F2 plants segregating for A2/a2 also showed no correlation between flower color and the CHS1 genomic fragment; in this case, the CHS1 fragments had sizes of 10.6 kb and 9.1 kb (Figure 5B). These data show that the structural gene encoding CHS1 mRNA does not segregate with either the a or a2 loci, indicating that these loci must regulate the gene expressing pCHS1 in trans.

**CHS2 Is Expressed in Root Tissue, Irrespective of the a or a2 Genotype**

The clone pCHS2 isolated from root tissue was used as a probe for another member of the CHS multigene family. S1 analysis was carried out using a uniformly labeled single-stranded CHS2 probe on the same preparation of RNA used in the previous S1 experiment (Figure 4). Again, this probe includes vector sequence to ensure that DNA:DNA hybrids can be distinguished from DNA:RNA hybrids after gel electrophoresis. The S1 analysis of root RNA showed that the full-length protected fragment was induced by CuCl2. In addition, several smaller fragments were protected, some of which were induced by CuCl2, as shown in Figure 7; there is some variation between the three lines in the sizes of some of the protected fragments. These additional fragments can be ascribed to regions of other CHS transcripts that contain regions of homology to CHS2 and are present in root tissue from all lines. They are unlikely to be due to 5' or 3' heterogeneity in transcription of a single gene because they range in size from almost full length (1.59 kb) to approximately 530 bp. They are most probably derived from transcription of other members of the CHS multigene family that share limited but strong homology with CHS2 (Figure 2). This was further confirmed by two-dimensional protein gels of CuCl2-induced and uninduced root tissue, which revealed the induction of multiple CHS proteins by CuCl2, analogous to those seen in bean (data not shown). Multiple CHS proteins previously have been detected in immature leaves of pea (Beerhues, Robenek, and Wiermann, 1988) and in other plant species (Grab, Loyal, and Ebel, 1985; Ryder et al., 1987; Beerhues and Wiermann, 1988; Beerhues et al., 1988).

These data suggest that neither the a nor the a2 locus has an effect on the CuCl2-inducible expression of CHS2 in root tissue. However, the line carrying the a2 mutation differs in the time course of CuCl2 induction of CHS protein in root tissue (data not shown). In addition, the level of CHS2 expressed at 12 hr in uninduced root tissue is higher in the line carrying the a mutation than in wild type or the line carrying the a2 mutation. It is not clear whether the quantitative and qualitative differences seen in the level of CHS2 induction, the difference in the time course, and the variation in the sizes of the protected fragments are due to differences in genetic background.

**A Subset of CHS2 Transcripts, CHS3, Are Detected in Petal Tissue**

S1 analysis of RNA isolated from petal tissue using pCHS2 as a probe showed that a subset of the CHS2 transcripts, which we have called CHS3, are partially protected by the CHS2 probe and are expressed in petal tissue of wild type and at a lower level in a mutants, but not in a2 mutants. In contrast to petal, CHS3 transcript(s) can be detected in roots of all genotypes. S1 analysis of RNA isolated from pooled petal tissue harvested from eight purple-violet-flowered or eight white-flowered plants segregating for
Figure 7. Expression of CHS2 and CHS3 mRNA in Roots and Petal Tissue Isolated from Wild Type (w-type), a Mutants (a/a), and a2 Mutants (a2/a2).

A full-length pCHS2 cDNA probe was uniformly labeled and hybridized to 10 μg of total RNA extracted from CuCl₂-induced (+) and uninduced (−) root tissue 12 hr and 21 hr after treatment, petal tissue, and pooled purple-violet (P) or white (W) petal tissue from an F₂ population segregating for either A/a (I) or A2/a2 (II). After S1 analysis, the sample was fractionated in a 3% polyacrylamide 8M urea gel. CHS3 fragments are indicated by arrows, and the full-length protected fragment by FL.

either A/a or A2/a2 gave the same result, showing that these differences were not due to a difference in genetic background (Figure 7, I and II). As discussed earlier, these transcripts probably arise from a different class of the CHS multigene family from that encoding CHS2. This indicates that the a locus quantitatively regulates the expression of CHS3 transcript(s) in petal tissue, whereas the mutation at the a2 locus blocks expression altogether.

It is not clear why the expression of CHS3 in petal tissue from a mutants does not lead to pigmented flowers. One possible explanation is that the mRNA is compartmentalized within cells or between cell layers (pigmentation is epidermal: Schmelzer, Jahnen, and Hahlbrock, 1988), rendering the protein unavailable for other steps in the anthocyanin pathway. Another possibility is that, because the 5' end of the transcript has a different sequence from that of CHS1, demonstrated by the lack of cross-protection of CHS3 by CHS1 in the S1 experiments, the amino acid sequence may be diverged enough to give this CHS a slightly different enzymatic activity from that of CHS1 and, hence, a different function. A third possible explanation is that the a locus, like the c1 regulatory locus in maize, may control other enzymes in the anthocyanin pathway in addition to CHS1, thereby creating blocks to pigment formation further down the pathway.

DISCUSSION

We have demonstrated that CHS in pea is encoded by a multigene family. It is not clear how many genes make up this family because the genomic EcoRI DNA fragments detected at low stringency could represent complete genes or fragments arising from genes that contained internal EcoRI sites. In bean, there are six to eight genes encoding CHS that are specifically induced by different stimuli (Bell et al., 1986; Ryder et al., 1987), suggesting that members of this multigene family are differentially expressed. In petunia, at least eight CHS genes exist, four of which appear to be differentially expressed in flowers and after UV light induction of seedlings and cell suspensions (Koes et al., 1989). In the case of pea, we have identified transcripts from three members of the CHS multigene family, CHS1, CHS2, and CHS3, that are differentially expressed. In petunia, at least eight CHS genes exist, four of which appear to be differentially expressed in flowers and after UV light induction of seedlings and cell suspensions (Koes et al., 1989). In the case of pea, we have identified transcripts from three members of the CHS multigene family, CHS1, CHS2, and CHS3, that are differentially expressed. CHS1 and CHS3 are expressed in both petal and root tissue, whereas CHS2 expression was detected only in root tissue.

We have shown that both the a and a2 loci of pea regulate CHS gene expression in trans (Table 1). Plants carrying a mutation at the a2 locus do not express CHS1 or CHS3 in petal tissue. In contrast, plants carrying a mutation at the a locus, although not expressing CHS1, do express CHS3, but at a lower level than wild type; this phenomenon is independent of genetic background. In roots, expression of all three CHS genes is induced by CuCl₂ regardless of the genotype at the a and a2 loci. There may be some differences in the time course of CHS induction and the quantity of CHS expression in plants carrying the a2 mutation as compared with wild type; however, these differences may result from differences in genetic background between the lines tested.
Multiple regulators of CHS have been described in other plants. In maize, there are four loci, c1, r, vp, and clf, involved in the regulation of CHS expression (Dooner 1983). Alleles at two of these loci have been isolated, C1 (Cone, Burr, and Burr, 1986; Paz-Ares et al., 1986) and two members of the R gene family (Lc and R-S) (Ludwig et al., 1989; Perrot and Cone, 1989). The products of these share amino acid sequence homology with an animal myb proto-oncogene in the case of two members of the CHS multigene family exhibit different complex kinetics and level of induction in response to elicitor (Ryder et al., 1987). A similar mechanism involving both common and spatially specific genetic signals has been proposed to explain how the regulatory gene delila interacts with a series of alleles at the pallida locus that confers diverse patterns of flower color in Antirrhinum majus (Almeida et al., 1989).

As far as we are aware, there are no other cases in which regulatory gene mutants have shown to affect the expression of distinct members of a multigene family in a spatially specific manner. There are several reasons why such genetic regulation may not have been studied. First, mutations in regulatory genes that affect the expression of individual members of a multigene family would have no observable phenotype if other members of the family were expressed at the same time and place and could, therefore, complement the lesion. Second, the phenotypic effects of varying the expression of many multigene families are ill defined. Third, mutations in regulatory genes may be lethal if their targets (e.g., rDNA, histones) are essential for viability or if the regulatory genes control other functions as well as the multigene family in question. Finally, many studies have focused solely on developmental or environmental regulation rather than seeking the underlying genetic basis.

In pea, individual members of the CHS multigene family can be activated selectively in hypocotyls by wounding, infection, and irradiation (Ryder et al., 1987). The authors propose that the differential accumulation of specific transcripts in response to different environmental stimuli could arise in part from selective transcription of specific CHS genes (Ryder et al., 1987). Our results suggest that the selective transcription of CHS genes in pea could be mediated by a combination of different trans-acting genes interacting with multiple cis-acting elements, allowing the regulation of expression of individual members or subclasses of the multigene family.

### METHODS

#### Pea (Pisum sativum) Lines

The purple-violet-flowered line JI 336 (wild type) and white-flowered line JI 1139 (a mutant) were obtained from the John innes germplasm collection. The white-flowered line T 234 (a2 mutant) was a gift from N.F. Weedon.
Protein was extracted from flowers by homogenizing 0.6 g, fresh weight/mL in 0.1 M phosphate buffer (pH 8) containing 4 mM 2-mercaptoethanol. The homogenate was spun in a microcentrifuge for 5 min, and the supernatant was used for immunoblotting. Proteins from equivalent amounts of fresh weight of tissue were separated by electrophoresis in a 15% SDS-polyacrylamide gel (Laemmli, 1970) and transferred to nitrocellulose filters according to Towbin, Staehelin, and Gordon (1979). The filters were incubated with rabbit antibody raised against CHS isolated from parsley and stained using alkaline phosphatase-conjugated anti-rabbit IgG according to Blake et al. (1984).

RNA Preparation

RNA was extracted from flowers according to Kloppstech and Schweiger (1976), except that total RNA was precipitated at 4°C with 2 M LiCl before application to oligo(dT)-cellulose columns.

DNA Analysis

DNA was prepared from pea as described by Ellis et al. (1984), digested with EcoRI or BamHI, and separated in 0.8% vertical agarose gels in 25 mM Tris-acetate buffer. Transfer to Hybond-N filters and hybridization were carried out according to Southern (1975), and the filters were washed twice for 30 min each under low stringency (2 × SSC, 0.05% SDS, 65°C), or high stringency (0.1 × SSC, 0.05% SDS, 65°C).

CuCl2 Induction of CHS

Copper chloride induction of CHS in radicles was carried out as described by Sweigard et al. (1988). Seeds were surface-sterilized in 5% sodium hypochlorite (10% to 14% w/v available chlorine), 80% ethanol for 5 min and washed in several changes of distilled water. The seeds were soaked overnight in 4 mL of water/g of seeds at 20°C in the dark. After removal of the excess water, the seeds were germinated for an additional 3 days. The germinated seeds were covered either with 5 mM CuCl2 or water for 1 hr, rinsed four times with water, and incubated further in the dark. Samples were taken 12 hr and 21 hr after treatment with CuCl2.

cDNA Libraries

Complementary DNA to isolated poly A+ RNA (10 μg) was prepared using the Amersham cDNA synthesis kit. The cDNA was either blunt-end ligated into Smal-digested pUC19 (Yanisch-Perron, Vieira, and Messing, 1985), or EcoRI linkers were added and the cDNA was ligated into the EcoRI site of the insertion vector λNM1149 (Murray, 1983). Plasmid libraries were screened by colony hybridization (Grünstein and Hogness, 1975), and phage libraries by plaque hybridization (Benton and Davis, 1977). The relevant recombinants were subcloned into M13mp18 or M13mp19 (Vieira and Messing, 1982), using the methods described by Maniatis, Fritsch, and Sambrook, (1982), and the 5' and 3' ends of each clone were sequenced according to Sanger, Nicklen, and Coulson (1977).

S1 Analysis

Labeled single-stranded DNA was prepared on the appropriate M13 template by the "prime cut" method (Hudson and Davidson, 1984). The M13 clones consisted of either the 5' region of pCHS1 cDNA cloned into the EcoRI-PstI site of M13mp18 or full-length pCHS2 cloned into the EcoRI site of M13mp19. Sequencing primer annealed to M13 DNA was extended with 2 units of Klenow polymerase, α-32P-dCTP (20 μCi), and unlabeled deoxynucleotide triphosphates (20 μM dTTP, dGTP, and dATP, 2 μM dCTP) for 20 min at room temperature and then chased with all four triphosphates (56 μM) for 20 min at room temperature. After heating at 65°C for 10 min, the DNA was cut with PvuII for 3 hr at 37°C. The labeled DNA was denatured by boiling and run in a 3% acrylamide gel to isolate the uniformly labeled single-stranded probe. After isolation of the probe from acrylamide, it was mixed with 10 μg of total RNA, phenol extracted, ethanol precipitated, resuspended in 10 μL of hybridization buffer [80% deionized formamide, 40 mM Pipes (pH 6.4), 400 mM NaCl, 1 mM EDTA] and incubated at 75°C for 20 min, after which it was transferred to a 50°C waterbath for 3 hr, diluted in 300 μL of S1 nuclease buffer [200 units/mL S1 nuclease in 280 mM NaCl, 30 mM Na acetate (pH 4.5), 4.5 mM Zn acetate], and incubated at 37°C for 90 min. The reaction was terminated by the addition of 75 μL of 2.5 M NH4-acetate and 50 mM EDTA, phenol extracted, and precipitated with tRNA (10 μg). The protected fragments were resuspended in 10 μL of water, 5 μL of which was lyophilized, and 3 μL of formamide dye was added. After boiling for 2 min, the sample was loaded onto a 3% polyacrylamide 8 M urea gel.

ACKNOWLEDGMENTS

We would like to thank Klaus Hahlbrock for the parsley CHS antiserum and for helpful comments, Chris Lamb for the bean CHS cDNA clone, and Chris Bell for technical assistance in the early stages of this work. We would also like to thank Peter Scott for photography and Roy Davies and David Hodgwood for their comments on the manuscript. The John Innes Institute is supported by a grant in aid from the AFRC.

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Identification and genetic regulation of the chalcone synthase multigene family in pea.
C L Harker, T H Ellis and E S Coen
*Plant Cell* 1990;2:185-194
DOI 10.1105/tpc.2.3.185

This information is current as of December 29, 2020

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