23 Mar 1994

Verification of the Isolation of the Chalcone Isomerase Gene from Glycine Max

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VERIFICATION OF THE ISOLATION OF THE CHALCONE ISOMERASE GENE FROM *GLYCINE MAX*

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ABSTRACT

In this paper we report the verified isolation of the gene encoding the flavonoid-biosynthetic enzyme chalcone flavonone isomerase (CHI) from *Glycine max* as well as the procedures used to generate the results. We began by electroeluting an 865 base pair (bp) *Hin*dIII fragment from pCHI1. We then restricted the pDR10 plasmid with *Hin*dIII. In order to test for homology, we hybridized the pDR10 fragment with nick translated pCHI1 fragment as a probe. For a second electrophoresis experiment, we cut pDR10 with *Eco*RI and *Hin*dIII. We also restricted several different pDR10 plasmids with a variety of deletions. After performing a Southern blot on the second restriction, we were able to determine that the homology for the CHI gene lies in a 1983 bp segment between the multicloning *Hin*dIII site and *Pst*I site of the insert.

INTRODUCTION

The chalcone flavonone isomerase gene is involved in the production of metabolic intermediates known as flavonones. Flavonones are responsible for a variety of biological activities including functions during pollination and seed dispersal, flower coloration, legume nodulation and protection from harmful doses of ultraviolet radiation and white light [1, 2, 3]. This particular gene encodes chalcone flavonone isomerase (CHI), an enzyme necessary for the interconversion of naringenin chalcone and naringenin, which is in turn converted into flavonoids, anthocyanins, and isoflavonoids (**Figure 1**) [2]. Flavonoids and anthocyanins are highly UV-absorbent and protect the plant DNA from the mutagenic effects of sunlight. One particular group of isoflavanoids, phytoalexins, play key roles in plant pathogen protection [2, 4].

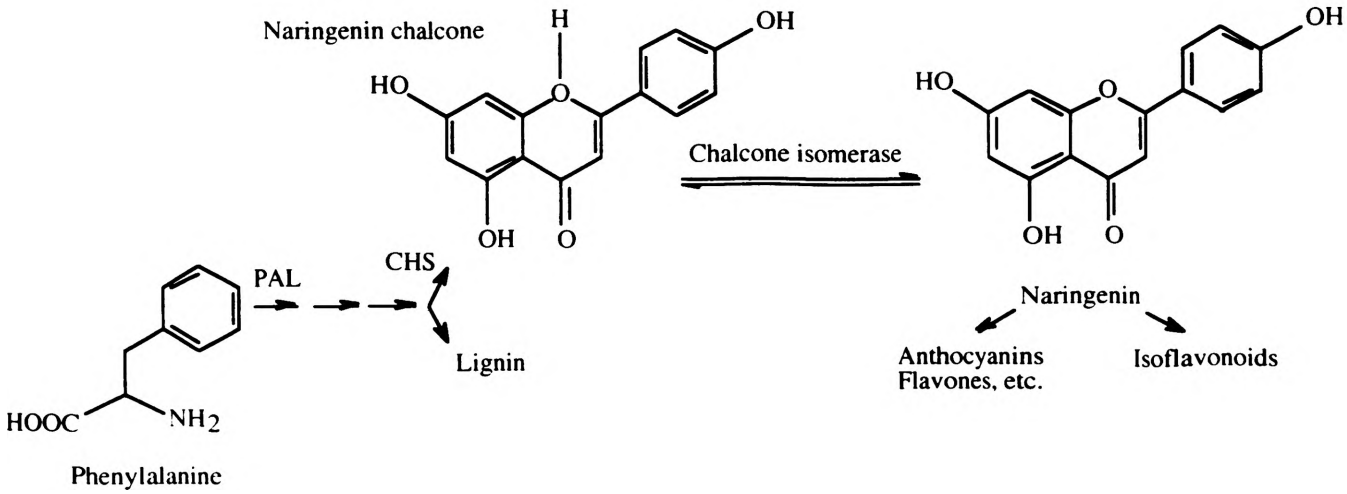

Figure. 1. Metabolic Role of CHI Gene in Phenylpropanoid Biosynthesis.

Previously, a soybean leaf genomic library in lambda Charon 35 was screened using the nick translated pCHI1 (*Phaseolus* CHI clone) plasmid without isolation of the insert cDNA away from other vector sequences. DNA was isolated from several positive recombinant phages, and Southern blots of restricted DNA were performed under similar conditions (entire vector as probe). The location of the putative soybean CHI was narrowed to a 3.9 kilobase (kb) *Hin*dIII fragment on the phage insert. This fragment was subsequently subcloned into pUC119 for nucleotide sequence determination and designated pDR10 (**Figure 2**). The purpose of the experiments reported here was to verify that the hybridization between the soybean DNA and pCHI1 was the result of homology to the *Phaseolus* gene-containing insert and not vector sequences.

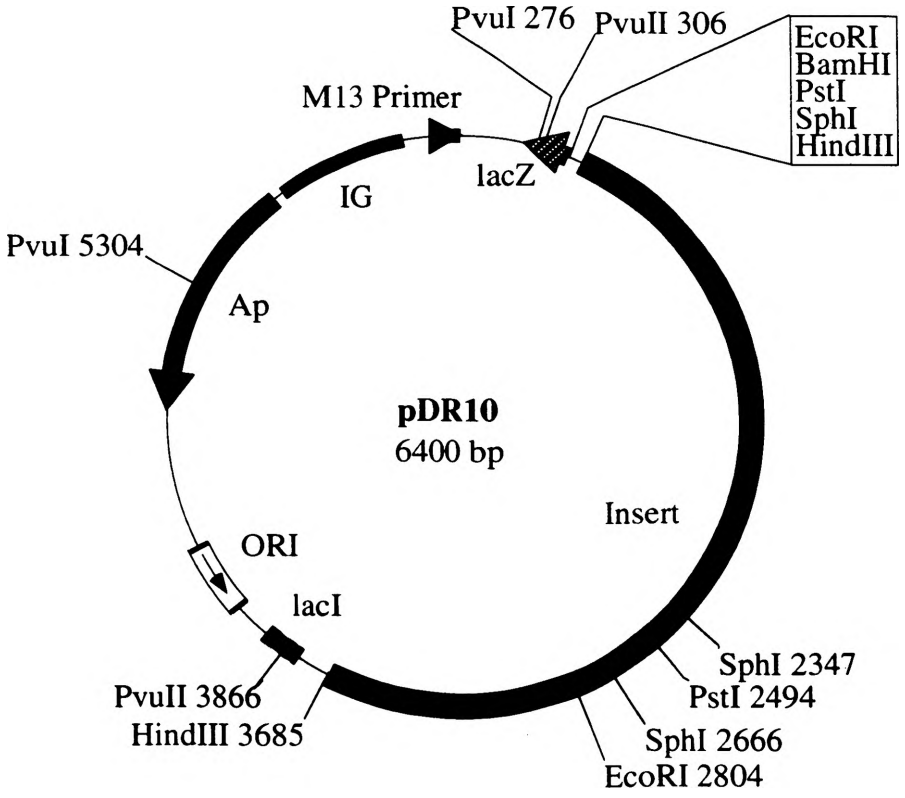

Figure. 2. Diagram of pDR10 Plasmid Showing Subcloned Insert.

RESULTS AND DISCUSSION

Results

Through our endeavors, we were able to produce two Southern blots identifying the location of the CHI gene on a 1983 base pair (bp) fragment located between the *Hin*dIII and *Pst*I sites of the inserted fragment of the pDR10 plasmid (**Figure 3**).

Methods of Determination

We digested the plasmid (pDR10) with *Hin*dIII restriction endonuclease, obtaining two fragments of approximately the same size: one containing approximately 3238 bp (soybean insert) and the other containing approximately 3121 bp (vector) (**Figure 4a, Lane 2**).

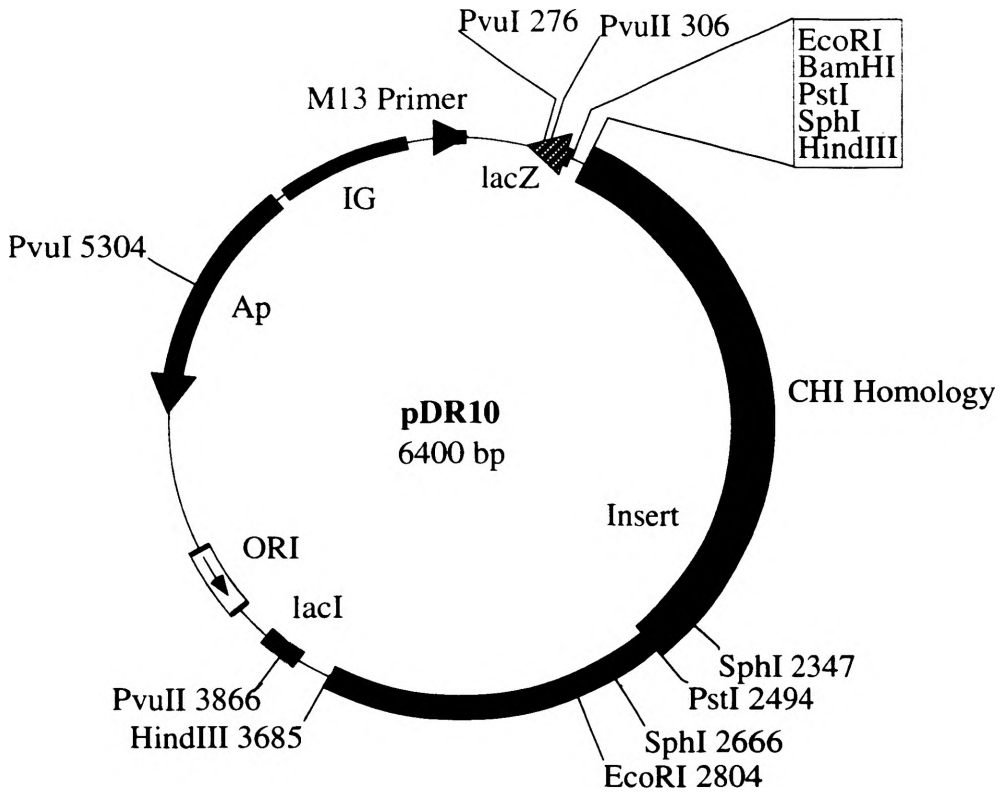

Figure. 3. Diagram of pDR10 Plasmid Showing Region of Homology.

On the Southern blot (**Figure 4b, Lane 2**), we were unable to identify the portion of the plasmid containing the gene because the similar size of the fragments caused them to run in close proximity on the gel. It is apparent from Figure 4b, lane 2 that only one band is hybridizing but without an appropriate control, we could not conclude that it was the band corresponding to the insert and not the vector. We then generated the second restriction, using the pDR10 plasmid digested with *Hin*dIII and *Eco*RI restriction endonucleases, producing three separate digestion fragments of distinctly different sizes: one containing approximately 3162 bp, a second composed of 2307 bp, and the third containing 931 bp (**Figure 5a, Lane 3**). On the second Southern blot we were able to clearly identify that the 2307 bp fragment (**Figure 5b, Lane 3**) contained the CHI homology.

Conclusions

The restricted DNA fragment that indicated homology lies between the larger and smaller bands allowing us to deduce the approximate location of the gene on the 2307 bp fragment. Another lane on the same gel, shows a *Hin*dIII/*Pst*I digestion of a plasmid that has the 1991 bp *Pst*I fragment removed. The observation that no hybridization occurred in this lane indicates that the area containing the CHI gene is a 1983 bp segment located in the region between the *Hin*dIII area of the multicloning site and the *Pst*I site on the inserted fragment (**Figure 3**).

Problems to be Addressed

There are, however, several different things we are unable to decipher from our results. We do not know if the entire gene is included on the 1983 bp strand. The pCHI1 fragment used as a probe is only composed of 86 % of the

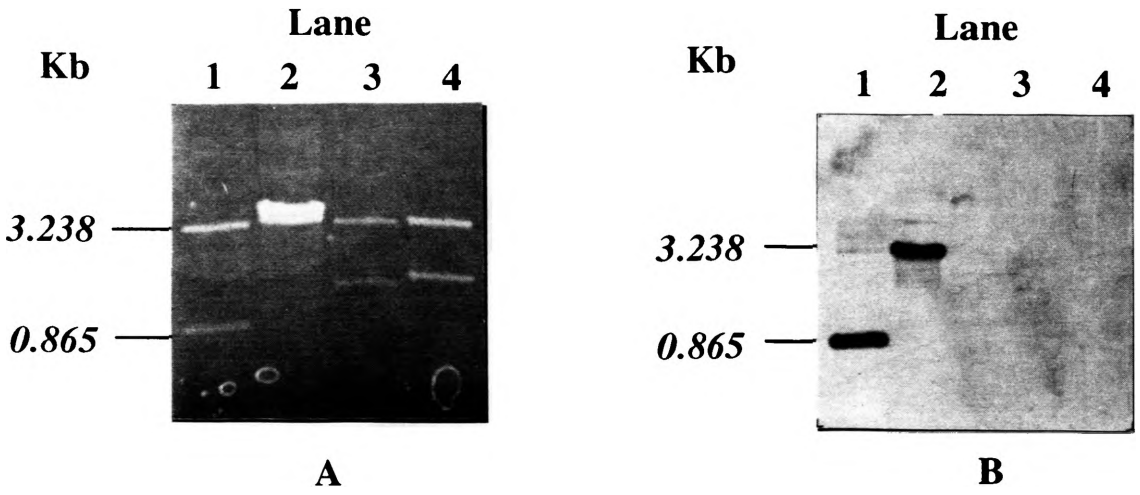

Figure. 4. Diagram of Electrophoresis Gel (A) and Corresponding Southern Blot (B).

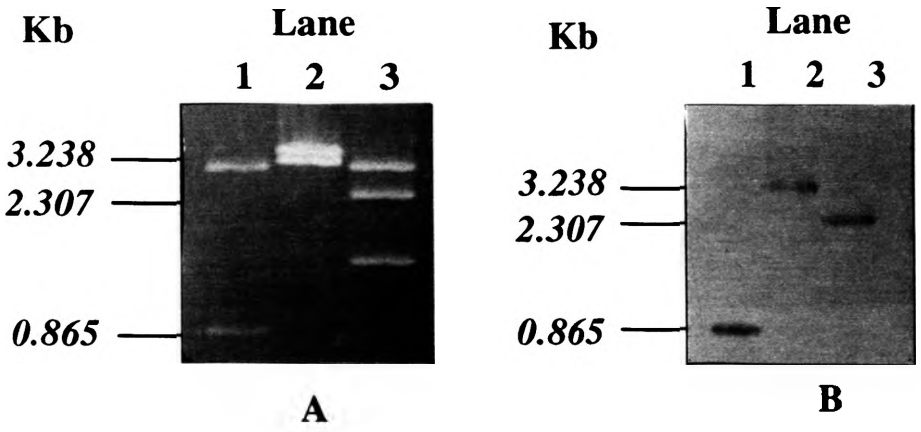

Figure. 5. Diagram of Electrophoresis Gel (A) and Corresponding Southern Blot (B).

entire CHI gene [4]. This means that there is 14 % of the gene that we are unable to account for. We are also unable to determine if any of the segment we have isolated includes one or more introns. In *Petunia hybrida*, two separate CHI genes have been isolated: CHI-A and CHI-B. The *P. hybrida* non-allelic CHI genes contain zero and three introns respectively [2]. In *Phaseolus vulgaris*, however, there exists a single CHI gene [2]. If the *G. max* CHI gene demonstrates similarity between the CHI-A of *P. hybrida*, the segment we isolated only contains coding sequences. Likewise, if the *G. max* CHI gene demonstrates similar structure to the CHI-B gene of *P. hybrida*, we have isolated a sequence containing at least one intron and quite possibly three. Since the pCHI1 fragment used as a probe was isolated from *P. vulgaris* and considerable homology was indicated by our experiments, we believe that the *G. max* CHI gene demonstrates several similarities to the CHI gene of *P. vulgaris*.

In order to answer these questions, we are currently working to sequence this gene in its entirety.

MATERIALS AND METHODS

Restriction Endonuclease Digestion and Agarose Gel Electrophoresis

Plasmids (pDR10) were digested with *Eco*RI, *Hin*dIII, *Pst*I, and *Sph*I and pCHI1 plasmids with *Eco*RI in a water incubator at 37 °C for twenty-four hours.

We then loaded the digestions into a 0.7 % agarose gel and applied 150 volts of current for one hour. The gels were then removed from the electrophoresis apparatus, stained using ethidium bromide, and photographed, as seen in figures 4a and 5a.

DNA Probe Preparation

Nick Translation Labeling

We mixed 1 µL of DNA, 6 µL of distilled water, 2 µL of 10X Buffer, 4 µL of dG/dA/dC mix (2 µL of each), 5 µL of biotin-11-dUTP, and 2 µL of Pol I/DNase I mix. We mixed it well by vortexing and incubated it at 15 °C in a water bath. After one hour, we removed the tube from the incubator, added 5 µL of stop buffer and mixed. We then added 75 µL of TNE (Tris-NaCl-EDTA). This mixture was loaded on top of the equilibrated spin column and centrifuged for five minutes at high speed.

Probe Purification by Exclusion Chromatography

We placed a small amount of glass wool into the bottom of 1 mL tuberculin syringe, forcing it to the bottom with the plunger. We then filled the syringe with G-50 Sephadex beads and placed it into a 15 mL Corex tube. At this time, we centrifuged the tube and syringe at high speed for five minutes. We repeated the previous two steps until the packed column reached the 1 mL mark on the syringe. We then loaded 100 mL of TNE on top of the packed column and centrifuged it for five minutes at high speed. We repeated this step a second time. We then placed a decapped Eppendorf tube into the bottom of a clean Corex tube and placed the syringe in the tube, with the bottom of the syringe inside the Eppendorf tube.

Southern Blotting

Transfer

Gels were treated for thirty minutes with 250 mL of 1.5 M NaCl/0.5 M NaOH with one change of solution after fifteen minutes. They were then soaked in 250 mL of 1.5 M NaCl/0.5 M Tris (pH 7.4) for thirty minutes with one change of buffer after fifteen minutes. The gels were then soaked in 6X SSC (0.9 M NaCl/90 mM Sodium Citrate) for fifteen minutes just prior to beginning the transfer procedure. We fabricated a wick (16 cm x 30 cm) from 3MM Whatman filter paper. We then cut three gel sized pieces (7 cm x 8 cm) of 3MM Whatman filter paper and one from nitrocellulose paper. We also cut approximately 7.5 cm of single fold paper towels. The wick was placed on the transfer apparatus and prewet with 6X SSC. The gel was placed, face (well side) down, on the wick. We prewet the nitrocellulose paper in distilled water then in 6X SSC. The nitrocellulose paper was then placed on top of gel ensuring that no air bubbles were trapped underneath. The three pieces of Whatman filter paper were prewet in 6X SSC and placed on nitrocellulose/gel stack. The stack was completed with 7.5 cm of paper towels. A small weight was added to ensure uniform transfer. Plastic wrap was placed over the exposed areas of the Whatman filter paper wick. After twenty-four hours, we disassembled the apparatus and blotted the nitrocellulose with 3MM Whatman filter paper. We then baked the nitrocellulose in an incubator at 80 °C for twenty-four hours.

Prehybridization

We prewet the baked filter in 6X SSC. The filter was then placed into a sealable bag and 10 mL of prehybridization buffer (50 mg Dry Milk/6 mL 20X SSC [3 M NaCl/0.3 M Sodium Citrate]/6 mL distilled water/8 mL Formamide) was added. We removed the trapped air in the bag, sealed it and prehybridized in a 37 °C water bath for twenty-four hours.

Hybridization

We added the nick translated probe DNA to 5 mL of hybridization buffer. We then denatured the probe DNA at 65 °C for ten minutes and quick cooled in ice/water. We removed the prehybridization buffer from the bag, added hybridization buffer, removed trapped air, and sealed. We then incubated the bag in a water bath for twenty-four hours at 37 °C.

Removing Excess Probe

We removed the hybridization buffer and washed the nitrocellulose three separate times in 100 mL of Primary Wash Buffer (2X SSC/0.1% Sarcosyl) for ten minutes each at room temperature with gentle tilting. We washed the filter with three separate treatments of 100 mL of Secondary Wash Buffer (0.1X SSC/0.1% Sarcosyl) for ten minutes each at room temperature with gentle tilting. We then washed the blot in 60 mL of Blocking Buffer (90 µL Tween 20/90 mL Conjugate Buffer) for two hours.

Binding of AP Conjugate

We rinsed the filters in 2X SSC. We then washed the filters in 30 mL of Blocking Buffer three separate times for five minutes each. Next, we incubated the filters in 10 mL of Conjugate Buffer/AVIDx-AP (0.2 g I-Block/0.02 g Sodium Azide/10 mL of 10X PBS/10 µL AVIDx-AP in 100 mL of distilled water) for thirty minutes with gentle tilting. The filters were then washed in 30 mL of Blocking Buffer for five minutes. We then washed the filters in 50 mL of Wash Buffer (0.072 g Sodium Azide/24 mL 10X PBS/0.72 mL Tween 20 in 240 mL of distilled water) four separate times for five minutes each. The filters were then incubated in 20 mL of Assay Buffer, pH 10 (0.025 g $MgCl_2$/0.025 Sodium Azide/1.2 mL DEA in 125 mL of distilled water) two separate times for two minutes each. They were then incubated in 20 mL of Assay Buffer/1 mL Sapphire mixture for thirty minutes with gentle tilting. We washed the filters in 30 mL Assay Buffer two separate times for five minutes each. We then added 50 µL of AMPPD to 5 mL of Assay buffer and incubated the filter in the mix for five minutes.

Chemiluminescent Detection

We drained the solution from the filter. We then placed the filter between two pieces of plastic wrap. The filter was exposed to X-ray film for five minutes in a light-tight cassette. We then developed the autoradiograph using standard procedures. The autoradiographs were then converted into photographs as can be seen in figures 4b and 5b.

ACKNOWLEDGMENTS

We would like to thank Darren Rice for his work in the production of the pDR10 plasmid and Dr. Christopher Lamb and the Salk Institute for generously providing the pCHI1 probe. I would also like to thank Dr. Ron Frank for designing and overseeing the project as well as for his patience and critical reading of this manuscript.

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