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Phenylalanine Ammooia-Lyase

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Abstract

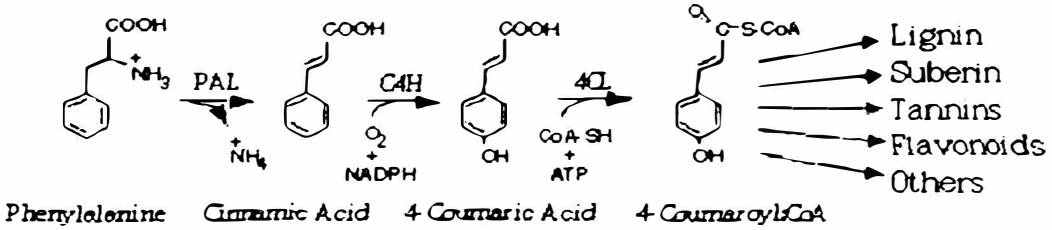
Phenylalanine ammonia-lyase (PAL), the first enzyme in the phenylpropanoid pathway, acts a key control point in this complex pathway and is known to consist of a gene family. The goal of this study was to isolate members of the PAL gene family using gene specific primers. But before this could be accomplished, controls had to be prepared using previously made primers and known PAL gene family members. The plasmid control, at approximately 500 basepairs, has been created and the genomic DNA control is still in the process of being worked out. Once the genomic DNA control has been finalized, gene specific primers will be ready to be used on genomic DNA.

Introduction

The phenylpropanoid pathway, found in plants, produces secondary metabolites that are not absolutely necessary for the plant to perform basic functions, but aid in the organism's survival. A complex metabolic system, the phenylpropanoid produces a vast array of products, including: isoflavanones, isoflavones, flavanoids, flavonols, anthocyanins, condensed tannins, lignin, sinapate esters, coumarins, and salicylic acid.[1] These products perform diverse functions, such as roles in plant defense, pigmentation, protection from ultraviolet light, recruitment of pollinators and seed dispersers, strengthening of cell walls, and signaling.[2] Phenylpropanoids *are not produced in all plant species, but hydroxycinnamic acid and flavanoids are found in most* higher plants. [1]

The phenylpropanoid pathway begins with the deamination of the amino acid L-phenylalanine via the enzyme L-phenylalanine ammonia-lyase (PAL) to trans-cinnamic acid (Figure 1). In many plant species such as tobacco and potato tubers this deamination step by PAL has been shown to be the rate limiting step, and therefore is a key control point in the phenylpropanoid pathway. [1]

Figure 1[3]



Many of the genes of the phenylpropanoid pathway, including PAL, are members of gene families, closely related genes that produce analogous products. [1,4] Multigene families of this pathway are of important biological significance because these families may be responsible for the independent regulation and production of end products in the same or different cells. For instance, in legumes a phenylpropanoid can be used as both a phytoalexin and a signal molecule to attract symbiotic microorganisms and would need to be regulated to meet the needs of the cell.

Moreover, gene families are responsible for gene dosage. For example, if a protein needed to be made quickly and in massive quantities, then providing many copies of the gene at key control points, such as PAL, would allow for the rapid production of the protein. [1]

The development of genomic sequencing technology and expressed sequence tag (EST) databases has broadened the depth of understanding of the complexity of gene families. By using BLAST search tools in various Plant Gene Index databases, such as TIGR (http://www.tigr.org), it possible to find sequences of members of a gene family or ESTs in a well studied species, for example soybean, *Glycine* max, and then use bioinformatics programs, for instance Gene Tool, to obtain the particular sequence information needed for a study. [1]

The goal of the research project was to isolate members of the PAL gene family in soybean using gene specific primers, designed from EST sequences from another student project. Before using new gene specific primers to isolate unique PAL genes, controls were created from previously designed primer and used to amplify previously isolated PAL genes in soybean in order to compare to the newly isolated genes.

Materials and Methods

Growth of colonies containing pP10ES1.2, pP10S3.3, and pP10ESs2.1

Competent cells that contained plasmids pP10ES1.2, that contained the 1.2 kilobasepair DNA fragment, and pP10S3.3, that contained the 3.3 kilobasepair DNA fragment, were plated on YT plates and incubated at 37C overnight.

The 2.1 DNA fragment was transformed into competent cells. Ten microliters of undiluted pP10ESs2.1 was added to 200 ul of thawed competent cells. The cells were left on ice for ten minutes, and then heat shocked at 42C in a water bath for 50 sec. The cells were put back on ice for 2 min, and then one ml of cold YT broth was added. The cell-broth mixture was incubated with shaking for 30-45 minutes at 37C. Following incubation, 100 ul of IPTG and 60 ul of X-gal were added to the mixture, and then 100 ul aliquots of the cell mixture were plated on YT plates. The plates were incubated overnight at 37C.

Colonies from plates of pP10ES1.2 and pP10S3.3 and white colonies from pP10ESs2.1 were individually inoculated into flasks containing 100 ml of YT Broth and five mg of ampicilan. The flasks were incubated with shaking overnight at 37C.

Plasmid DNA Isolation

The following solutions needed to be prepared immediately prior to isolation:

Lysozyme:	4mg/ ml of wash buffer
NaOH/SDS:	200 mM NaOH
	1% SDS
K/Acetate:	2.5 M Potassium
	2.0 M Acetate

Five ml of the culture were transferred to 15 ml sterile centrifuge tubes and centrifuged at high speed for ten minutes. Two-five milliliter samples were prepared for each plasmid. The

supernatant was decanted, and the pellet was resuspended in one ml of wash buffer. The resuspension was transferred to sterile 1.5 ml snap cap microcentrifuge tubes and centrifuged for one minute at low speed. The supernatant was decanted into the original fifteen ml tube, and the last few microliters of supernatant were removed with a micropipette. The cells were resuspended in 100 ul of lysozyme, vortexed, and stored five minutes at room temperature. Two-hundred microliters of freshly prepared, ice cold NaOH/SDS solution was added to the resuspension and then mixed thoroughly by inversion. The mixture was kept on ice for five minutes, and then centrifuged at high speed for five minutes. With a Pasteur pipette, the supernatant was transferred to a new sterile 1.5 ml microcentrifuge tube. Two-hundred microliters of equilibrated phenol and two-hundred microliters of chloroform/isoamyl alcohol were added to the supernatant and thoroughly vortexed. The tubes were centrifuged on high speed for two minutes. Following centrifugation, the top-aqueous layer was transferred to a fresh 1.5 milliliter microcentrifuge tube. Eight-hundred microliters of ice cold 95% ethanol was added to the aqueous layer to precipitate the nucleic acids. The tube was vortexed and allowed to stand a room temperature for two minutes, followed by centrifugation at high speed for ten minutes. The supernatant was decanted and 500 microliters of 80% ethanol was added to the pellet and mixed by inversion. The mixture was centrifuged at high speed for five minutes, decanted, and the tube was allowed to drain by inversion on a Kim wipe. The pellet was thoroughly dried by leaving the microcentrifuge tube opened on its side resting on a Kim wipe overnight. The next day the dried pellet was resuspended in 100 microliters of TE and ten microliters of RNase, and stored at 4C until ready to be used.

Restriction Enzyme Double Digest of Plasmid DNA with Eco RI and Sac I

Both of the samples for each of the three plasmids were subjected to a double digestion. To each tube, the following was added: three microliters of appropriate plasmid DNA (pP10ES1.2, pP10s3.3, or pP10ESs2.1), 13 microliters of water, two of Eco 10x buffer, one microliter of Sac I, and one microliter of Eco RI. The contents were mixed and spinned, and then overlaid with thirty microliters of mineral oil. The digestion mixture was placed into a thermal cylcer overnight at 37C. The digestion reaction was stopped by adding five microliters of 5X Dye to the tubes and then stored at 4C until ready to be used. The digestion fragments were checked on a 0.7% agarose gel, using TAE buffer.

PCR on pP10s3.3

PCR with old reactants

Into a 0.5 microliter snap cap tube, the following reactants were added: four microliters of 10X Taq polymerase buffer, four microliters of 1:100 dilution of dNTPs, five microliters of 1:20 dilution of 010 primer, five microliters of 1:20 dilution of 043 primer, and one microliter of pP10s3.3 plasmid DNA. The contents of the tube were mixed and spinned. The tube was placed into a Thermal Cycler for 5 minutes at 94 C. One microliter of Taq polymerase was added and the tube was mixed and spinned. The mixture was overlaid with 30 microliters of mineral oil, and placed in the thermal cycle for 30 cycles, two minutes 94C for denaturation, two minutes 60C for primer annealing, and one minute 72C for polymerase extension. The PCR product was checked on a 2% NuSieve 3:1 Agarose gel with TAE buffer.

PCR with 2X Master Mix and Diluted Template
Into a 0.5 microliter snap cap tube, the following reactants were added: 12.5 microliters of 2X PCR Master Mix, 2.5 microliters of 1:20 dilution of 010 primer, 2.5 microliters of 1:20 dilution of 043 primer, one microliter of appropriate plasmid DNA pP10s3.3 (1:10 dilution or 1:100 dilution), 6.5 microliters of nuclease-free water. Two reactions were set up, one with a 1:10 dilution of plasmid template and the other with a 1:100 dilution of plasmid template. The contents of the tube were mixed and spinned. The tube was placed into a Thermal Cycler for 5 minutes at 94 C. One microliter of Taq polymerase was added and the tube was mixed and spinned. The mixture was overlaid with 30 microliters of mineral oil, and placed in the thermal cycle for 30 cycles, two minutes 94C for denaturation, two minutes 60C for primer annealing, and one minute 72C for polymerase extension. After the cycles were completed, 6.22 microliters of 5x Dye was added, and the PCR product was checked on a 2% NuSieve 3:1 Agarose gel with TAE buffer.

PCR with 5X Go Taq Buffer and 1:10 diluted Plasmid Template
Into a 0.5 microliter snap cap tube, the following reactants were added: Four microliters of 5X Green Go Taq buffer, thoroughly thawed and vortex, four microliters of 1:100 dilution of dNTPs, 2.5 microliters of 1:20 010 primer, 2.5 microliters of 1:20 dilution 043 primer, one microliter of 1:10 dilution pP10s3.3 plasmid DNA, and five microliters of nuclease free water. The contents of the tube were mixed and spinned and one microliter of Go Taq polymerase was added, followed by additional mixing and spinning. The mixture was overlaid with 30 microliters of mineral oil, and the above PCR program was ran. The PCR product was checked on a 2% NuSieve 3:1 Agarose gel with TAE buffer.

PCR with new Nucleotides
The PCR Reaction Mixture was performed as the above 5X Go Taq Buffer procedure except one microliter of new dNTP mixture and eight microliters of nuclease free water were added to the tube.

PCR on Genomic DNA from Soybean Leaf Tissue

Soybean seeds were planted and grown to maturity. Leave samples were collected. Extraction was performed with and following the directions of Cartagen Rapid Homogenization Kit: Plant Leaf DNA Amplification. Dilutions of 1:10, 1:100, and 1:1000 of the genomic DNA extractions were made and tried in the PCR reaction. The following reactants were added into 0.5 microliter snap cap tube: four microliters of 5X Green Go Taq Buffer, four microliters of 1:100 dilution of dNTPs, 2.5 microliters of 1:20 dilution of 010 primer, 2.5 microliters of 1:20 dilution of 043 primer, one microliter of the appropriate dilution of genomic DNA (1:10, 1:100, or 1:1000), and 5 microliters of nuclease free water. The contents of the tube were mixed and spinned. One microliter of Go Taq Polymerase was added to the tube, and it was mixed and spinned again. No mineral oil was added and it was run through 30 cycles of PCR. The PCR product was checked on a 2% NuSieve 3:1 Agarose gel with TAE buffer.

Another genomic DNA isolation method was used using DNAzol. The soybean leaves were torn into 1 cm2 pieces until 0.4-0.5 grams of tissue were weighed out. A mortar and pestle was used

to grind the leaves, and 1.2 ml of DNAzol buffer was added to the mortar while grinding continued until a homogenous slurry was obtained. The slurry was distributed between two 1.5 ml snap cap microcentrifuge tubes and stored at room temperature for five minutes with occasional mixing. Following incubation, 600 microliters of chloroform: isoamyl alcohol (24:1) was added to each tube, and extracted by vortexing the tube and centrifuging for ten minutes on high speed. The top aqueous layer of each was transferred to a clean 1.5 ml microcentrifuge tube, and 450 microliters of cold 95% ethanol was added and gently mixed to precipitate the nucleic acids. The tubes were allowed to incubate at room temperature for five minutes, and then centrifuged for five minutes on high speed. The supernatant was discarded and 600 microliters of wash buffer (five parts DNAzol:four parts ethanol(v/v)) was added and then swirled five minutes to resuspend the pellet. The tubes were centrifuged at top speed for five minutes, after which the supernatant was discarded. The pellet was briefly air dried, resuspended in 0.5 milliliters of ice cold 80% ethanol, and centrifuged at high speed for five minutes. The supernatant was discarded and the pellet was allowed to air dry overnight, after which the pellet was resuspended in 70 microliters of TE and stored at 4C.

Results and Discussion

The aim of this study was to isolate members of the gene family PAL from soybean with gene specific primers. In order to show that the DNA isolated with the gene specific primers is unique PAL genes, controls needed to be prepared from primers designed to amplify known PAL genes for comparative analysis. These previously isolated PAL gene included pP10ES1.2, marked by a 1.2 kilo basepair fragment, pP10S3.3, characterized by a 1.2 and 2.1 kilo basepair fragment, and pP10ESs2.1, designated by a 2.1 kilo basepair fragment when the plasmid was digested with Eco RI and Sac I.

The known genes needed to be transformed into bacterial cells for further genetic manipulation. The pP10ES1.2 and pP10S3.3 were already in bacterial cells, so these cells were grown on plates and the cells formed colonies, so the cells were still viable. However, pP10ESs2.1 was not in cells, so it was transformed into competent E. coli cells and IPTG and X-Gal were used as markers to ensure that the cells had taken up the plasmid and that the plasmid contained that the PAL gene. Colonies were identified and the cells allowed to multiply so that plasmid DNA isolation could be performed. Plasmid DNA was successfully extracted from all of the cell, but when a restriction double digest was performed with Eco I and Sac I, pP10ES1.2 had a 2.1 kilo base pair fragment instead of a 1.2 kilo basepair fragment, so it was actually pP10ESs2.1. Therefore, pP10ES1.2 plasmid had to be transformed into competent cells and another plasmid cell DNA extraction performed. After restriction double digest of pP10ES1.2, it was seen to contain a 1.2 kilo basepair fragment, so the transformation had been successful.

Next, PCR was performed on pP10S3.3 using previously designed primers. The first PCR reaction contained undiluted template and the results showed that the template had been amplified and not the desired DNA region, so a 1:10 dilution of the plasmid DNA was performed. Moreover, the PCR reactants were old so a new PCR Master Mix was purchased and used in the next reaction. However, no amplified regions were seen on the gel. Next, the 5X Green Go Taq Buffer protocol was performed. From this, a faint band was seen at approximately 500 base pairs. Because only a faint band was obtained a new nucleotide mixture was used in the PCR reaction in hope of better results, however, no band was seen. The PCR procedure was repeated again with the old nucleotides and during this attempt it was seen that

the thermal cycler, in which was used to carry out the PCR reaction, had stopped working. Therefore, the PCR reactions were carried out again with the old nucleotide and the new nucleotide mixture in a different thermal cycler. This time the gel showed a distinct bright band at approximately 500 base pairs with the old nucleotide mixture, but no bands with the new nucleotide mixture. Therefore, the old nucleotide mixture would be used to carry out amplification of the plasmid DNA control.

Since the desired region was amplified on the plasmid DNA, PCR was performed on the extracted genomic DNA. The first DNA extraction with the Cartagen kit yielded no PCR product. But because the DNA solution had been kept in the freezer for awhile before use in the PCR mixture, inhibitors not cleared up from extraction could of impeded the PCR reaction. Accordingly, the Cartagen DNA extraction method was performed again immediately followed by PCR. Nevertheless, there were no PCR products. The extraction method may have been too simplified and did not purify the solution containing the genomic DNA enough to be suitable for PCR. Therefore, a more involved genomic DNA extraction was performed using DNAzol. However, this method did not produce any genomic DNA and still had a large amount of contaminating polysaccharides. The DNAzol method will be attempted again with added extraction steps in hopes of ridding the genomic DNA of these polysaccharides. Once purified genomic DNA has been obtained, the primers 010 and 043 will be used to amplify the PAL region on the DNA.

After the controls are prepared, then the gene specific primers from the other student's project can be applied to the PCR on the genomic DNA in place of the 010 and 043 primers to hopefully amplify a new PAL gene family member.

The isolation of PAL gene family members is of biological significance because the enzyme is an important control point in the phenylpropanoid pathway, which produces many principal secondary metabolites in plants. These members of the PAL gene family can give us a better understanding of how this pathway is regulated in the same and different cells to maintain the cells needs.

Nomenclature

Gene family- "Group of closely related genes that make similar products" [4]

Polymerase Chain Reaction (PCR)- - "A technique whereby targeted regions of DNA are amplified. Short single stranded sequences of DNA known as primers, which bind to a complementary sequences and initiate extension of the adjacent DNA regions using DNA polymerase, are employed along with the thermophilic bacterium Thermus aquaticus which is resistant to denaturation at extremely high temperatures. Double stranded DNA is denatured to single strands to which the primers anneal at lower temperatures, and this is followed by primer extension resulting in a double stranded copy of the target sequence. This cycle involves strict control of temperature changes, in order for denaturation, annealing and polymerisation to occur, and is generally repeated thirty or more times in order to yield a large number of copies of the target DNA sequence."[5]

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