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Insertion of the Enzyme Cyclopropane Fatty Acid Synthase into Plastids through Agrobacterium Mediated Transformation

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Insertion of the Enzyme Cyclopropane Fatty Acid Synthase into Plastids through *Agrobacterium* Mediated Transformation

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Introduction

Transgenic plants are plants that have been genetically modified for increased usefulness to people. Essentially, a gene from one organism is placed into a target plant by a vector, often with *Agrobacterium tumefaciens*. Under natural conditions, *Agrobacterium* is a plant pathogen that infects plants by inserting genes into the plant's genome that are desirable to the bacterium itself. A tumor is then formed and the infected plant tissue makes molecules that the bacterium uses as food. It is possible to change this bacterium from a pathogen to a beneficial organism by removing the genes that it typically places into the plant's genome and replacing them with genes that will elicit desirable effects. In this project, I will attempt to make transgenic tobacco cells that will produce large amounts of dihydrosterculate, a cyclopropane fatty acid which could be used in industry as an effective high-temperature lubricant.

A fatty acid is a carboxylic acid with a chain of carbons named for the number of carbons in the chain and the number of double bonds located between carbons [15]. Dihydrosterculate is made when an eighteen carbon fatty acid with one double bond (18:1), reacts in the active site of the enzyme cyclopropane fatty acid synthase (CFAS). CFAS causes a structural change in 18:1 by adding a methylene group (CH_2) from S-adenosylmethionine, a molecule that often acts as a methyl donor. This forms a ring in the middle of the carbon chain and thereby producing the target molecule, dihydrosterculate.

CFAS was first cloned by Grogan and Cronan [6] and its amino acid sequence was first deduced by Wang *et al.* [20]. In nature, the molecule dihydrosterculate is not a product of *E. coli*'s CFAS catalyzed reaction. General cyclopropane fatty acids (CFA) are made by a transfer of a methylene group from S-adenosylmethionine to a *cis* double bond in the fatty

acid tail of an unsaturated fatty acid, in the same manner as the formation of dihydrosterculate [20]. In *E. coli* dihydrosterculate is not a product of the CFAS catalyzed reaction, lactobacillic acid is. However, it has been shown that the enzyme will catalyze the reaction from oleate to dihydrosterculate [16]. Plants tend to use a large amount of oleate as a precursor molecule for other fatty acids, so the necessary substrate for CFAS is present in plants. Oleate is also often associated with phosphatidylcholine, a phospholipid that CFAS can bind to and react with. The fatty acid substrates of CFAS are found in membrane bilayers. Given that CFAS is a water soluble protein, it shouldn't be able to interact very well with the hydrophobic inner layer of a lipid bilayer, nonetheless it does. For this to occur, a specific orientation of acyl chains need to be present in the membrane that favors the binding of the enzyme [7]. The enzyme binds to the bilayer and catalyzes its reaction on both sides of the layer [6]. It has been shown that CFAS only binds to phospholipids that are unsaturated or have cyclopropanes in the tails [17]. The exact chemical mechanism of the catalyzed reaction is still not well understood. A recent simplified model for the reaction is shown in Diagram 1 [5], but the specifics are still vague.

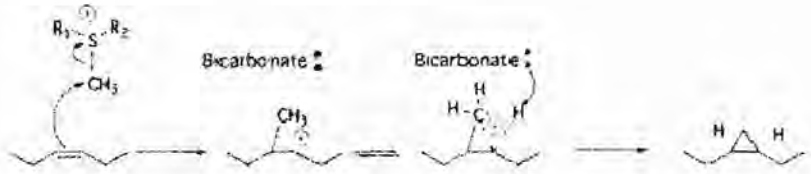

Diagram 1: The *cis* double bond reacts with the methyl group of S-adenosylmethionine. It is generally thought that a carbocation intermediate is involved in the mechanism. Bicarbonate has been shown to be important to the fast conclusion of the reaction, and its putative role is shown as deprotonating the methyl group. This diagram was obtained from Courtois and Ploux [5].

The purpose of CFAS in bacteria is unclear. It has been hypothesized that CFAs have some function related to cell stress. CFAs may be important to long-term survival of cells

that have gone into stasis. The evidence for this is that the *E. coli* CFAS gene has two promoters, one of which is a typical promoter and the other which is designed to be used by a special RNA polymerase partially coded by *rpoS*. *rpoS* encodes the sigma factor of the RNA polymerase that is used to turn on genes needed for long term survival of the cell while it is not growing [7].

However, crop plants do not naturally make the enzyme cyclopropane fatty acid synthase, at least not in amounts high enough to produce a significant amount of dihydrosterculate. Low levels of dihydrosterculate production have been observed when either a plant [2] or bacterial [14] gene for CFAS was introduced into the cytosol, or cellular fluid, of a tobacco plant. One possible reason for these low level yields is the lack of the 18:1 in the cytosol. In plants, 18:1 is synthesized in the plastids, plant organelles which can be used to store energy. Although some 18:1 is exported to other compartments, much of it is rapidly converted into 18:2, which cannot be used to make dihydrosterculate, therefore making it useless to this project. To account for this, bacteria and then tobacco will be transformed with a recombinant gene made to target CFAS to the plastid.

Transformation is the uptake of plasmid DNA by a bacterium from its environment. This phenomenon was first suggested by Griffith [9] through his observation that a heat killed virulent bacterial strain would turn a living avirulent strain virulent. Since the virulent bacteria were killed, the avirulent strain must have picked up something from the dead bacteria to change their phenotype, which turned out to be DNA. However, it was not Griffith who discovered that DNA was indeed the transforming principle. Avery, MacLeod, and McCarty [1] isolated different compounds, such as DNA, protein, and RNA to determine which the transforming principle was. Only the solutions that contained DNA showed

transformation. Transformation can be performed in the lab with specific cells and DNA so that DNA of interest, rather than random plasmids found in the extracellular environment, is inserted into the bacteria. *A. tumefaciens* can be used to transform plants, a much easier method than attempting to make plant cells take up plasmids themselves.

The discovery of *Agrobacterium* as a method to genetically modify plants came about as an accident, essentially. *Agrobacterium* causes growths to form on plants that are similar to tumors. Cancer research was being performed using *Agrobacterium*, which is how its unique abilities were discovered. There were no breakthroughs for cancer research, but there were benefits to researching this specific bacterium. Chilton *et al.* showed in 1977 that the tumorigenesis caused by *Agrobacterium* was actually due to a transfer of part of the tumor-inducing (Ti) plasmid naturally found in the bacterium [4]. This plasmid was found by Larebeke *et al.* to be the factor that caused virulence. It was later found that a specific region of the plasmid, called T-DNA, was the section inserted into the plant genome [8]. This was a great discovery, because it made it easy to remove the T-DNA and replace it with the gene that is being studied. This way, tumorigenesis would not occur, but specific DNA would still be transferred to the plant.

Targeting of proteins to the plastids is mediated by specific cleavable protein segments called transit peptides. In this case, CFAS needs to be targeted to the plastid, so a DNA segment encoding a transit peptide must be isolated that signals movement to the plastid during translation. Acyl carrier protein has such a transit peptide which can be utilized by attaching it to the N-terminus of CFAS of *Escherichia coli* [20], a common bacterium, which results in a recombinant gene. This recombinant gene will then be inserted into a plasmid which is a ring of DNA that can be reproduced in bacteria independently of

the bacterial genome. For plasmids to be useful in molecular biology there needs to be a method to distinguish bacteria that successfully take up the plasmid and those that do not. The method most commonly used is to have a gene in the plasmid code for a specific antibiotic resistance. The transformed bacteria are grown on medium containing the specific antibiotic. Only those colonies that survive should have the plasmid because of the resistance given by the plasmid. Plasmids also usually contain a multiple cloning site (MCS) where it is simple to use restriction enzymes to insert foreign DNA.

A restriction endonuclease is an enzyme that cleaves at a very specific site in a DNA sequence. The recognition sequence can be four to eight nucleotides in length. The longer the restriction site, the more specific the restriction enzyme is because there is less likely to be eight specific bases in a specific order then there is to be four. Restriction enzymes can leave either blunt ends, which contain no unpaired bases, or sticky ends, which have unpaired bases. The sticky ends allow for easy, specific cloning to take place. A common method for introducing a gene into a plasmid is called directional cloning. In directional cloning, both the gene and the plasmid are digested with two different restriction enzymes. The restriction site at the 5' end of the gene matches the restriction site of the plasmid closest to the promoter to be utilized, and therefore, the sticky ends created are complementary. The 3' restriction site on the gene is further away from the promoter in the MCS. This is a common practice because a gene is useless if inserted backwards because no functional protein is made. One reason that directional cloning is possible is because, during PCR, a primer can be used that contains a restriction site. This allows placement of very specific restriction sites at both ends of the gene of interest that would not normally be there. A strong promoter is located near this site to express the gene of interest in large amounts.

Another important enzyme utilized is ligase. Ligase actually catalyzes the reaction that connects the backbone of two different pieces of DNA. Sticky ends made by restriction enzymes do bond together, but the backbone doesn't, just the bases. Ligase takes the separate pieces and, by utilizing ATP, joins them together through a phosphodiester linkage, which is typical of nucleic acids. Ligase requires that at least one of the two pieces to be put together have a phosphate group, otherwise the chemistry (shown in Diagram 2) won't work.

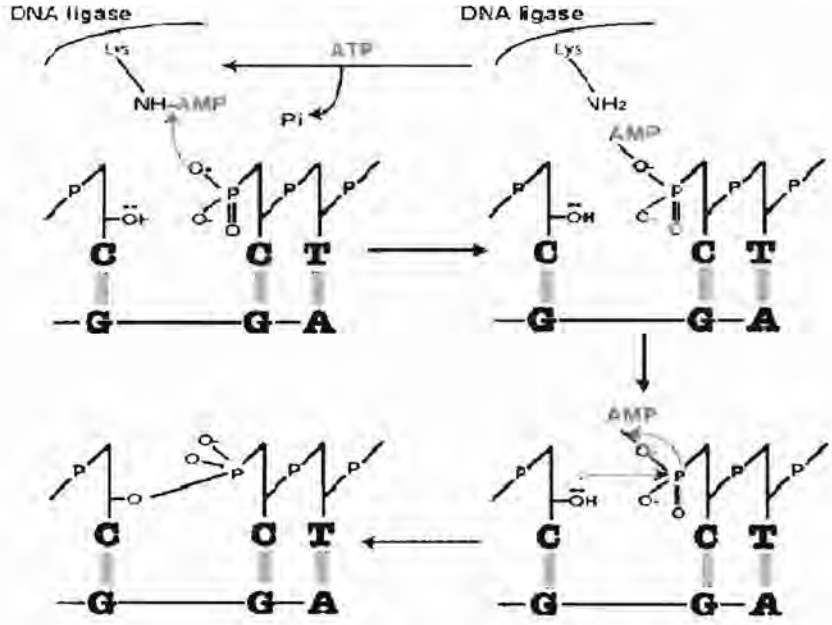

Diagram 2: This diagram illustrates the reaction mechanism of the enzyme DNA ligase. A phosphate from one of the two strands of DNA nucleophilically attacks an adenosine monophosphate (AMP) attached to a lysine residue on the enzyme in an addition-elimination reaction. Another addition-elimination reaction, with a hydroxyl on the opposite strand as the phosphate acting as the nucleophile, causes the release of AMP and the covalent attachment of the two strands [18].

Sometimes calf intestinal alkaline phosphatase is used on the plasmid before ligation to remove the end phosphates so that the plasmid cannot close on itself during ligation. This helps ensure that the ligation connects the plasmid to the gene of interest. Having different

restriction sites helps with this because it reduces base-pairing, but dephosphorylating the plasmid increases the chance of successful integration.

However, before the ligation takes place, the restriction endonucleases need to be removed from the DNA so that they would not continue to cut the DNA after it gets ligated together. Ligase takes ATP to function, so its ability to catalyze the reaction is limited. Restriction enzymes do not need anything besides magnesium ions. The magnesium ions are not altered in any way, so the reaction can be catalyzed as long as the protein is functional. Therefore, ligase will run out of ATP and the restriction endonucleases will keep the DNA cut apart. The purification of the DNA away from the enzymes is done by gel electrophoresis. The cut plasmid and the cut gene would be run on a gel in large quantities and then cut from the gel and placed gel purified. The enzymes will have been separated from the DNA by gel electrophoresis, so the DNA will not be contaminated and ligation can safely take place. This method is also used to separate the DNA of interest from the complementary pieces that could potentially be ligated back on.

There are a number of characteristics of DNA that are often utilized in molecular biology in addition to its ability to hydrogen bond with complementary strands. Gel electrophoresis, an extremely important method used as an analytical tool and as a method of purification, is possible because DNA is negatively charged. Furthermore, DNA is charged uniformly across its length. An agarose matrix forms the gel and the matrix has varying pore sizes. Smaller pieces of DNA will travel faster through the network of pores than larger pieces. The source of the movement is an electrical current. The DNA is loaded near the negatively charged end of the gel and travels towards the positively charged end. A ladder, or mixture of polynucleotides of known length, is loaded on a gel to determine the DNA of

interest's size. This method works because a one hundred base pair segment of DNA will travel the same distance as any other one hundred base pair segment on the same gel.

Another use for the negative charge of DNA is to purify the DNA using ion-exchange chromatography. Ion-exchange chromatography is the process of isolating DNA from buffer and contaminants by binding it to a positively charged resin. Because DNA has a very strong negative charge, it binds strongly to a positive resin. A buffer is run through the resin that is less positively charged than the resin, which helps elute off bound contaminants, but not the DNA. A strongly positive charged buffer, called an elution buffer, can be used to remove the DNA from the resin into a clean tube away from the contaminants that were previously present.

Another important characteristic of DNA is its ability to absorb in the UV spectrum due to the aromaticity of the purine and pyrimidine rings. It is possible to quantify the DNA using Beer's Law (Equation 1) and the absorbance at 260 nm.

$$A_{260} = Extinction\ coefficient\ x\ path length\ x [DNA]$$

Equation 1: Beer's law. The extinction coefficient is constant for a substance. The path length depends on the cuvette, but is often 1cm.

Another helpful aspect of the spectroscopy is that it is possible to roughly quantify the purity of the DNA based on the ratio of the absorbance at 260 nm to 280 nm. Very pure DNA should have a ratio of about 1.8 : 1. The extinction coefficient for DNA that consists of an equal number of each type of base is 0.020 $\mu g^{-1} mL\ cm^{-1}$ at 260 nm and a pH around 7. The path length of a typical cuvette is 1 cm [12]. Glass absorbs at 260 nm so either a quartz cuvette or a UV transparent plastic cuvette must be used to obtain an accurate absorbance.

Also, a standard is taken with whatever the sample is dissolved in to remove its reading from the final scan. An ideal absorbance reading is between 0.1 and 1.0. Past these values the reading might be inaccurate; therefore a dilution may need to be used. The final [DNA] must then be multiplied by the dilution factor.

A. tumefaciens uses a specific type of plasmid for infecting plants, so a recombinant gene made in *E. coli* needs to be inserted into this specific type of plasmid, which is then inserted into *A. tumefaciens* that can then insert it into a plant's genome, hopefully giving the desired effect. Once the *A. tumefaciens* inserts the promoter/transit peptide/CFAS sequence into the genome of the tobacco plant, the plant will produce the polypeptide sequence that corresponds to the recombinant gene. The polypeptide sequence is the straight, nonfunctional amino acid chain which contains the code for the transit peptide and CFAS. The transit peptide is made when the first portion of the polypeptide sequence is translated from RNA. The synthase enzyme portion of the polypeptide sequence is translated next. Then the plastid transit peptide causes this polypeptide chain to be led to the plastid [13]. To prevent the natural folding of the polypeptide chain into a functional protein, other proteins, called chaperones, keep it linear. The purpose of this is to make movement across the plastid membrane much easier than it would be with a complicated three dimensional structure. The transit peptide is then cleaved off as it enters the plastid in order to avoid altering the final structure, and therefore the function, of the protein.

Single-extension overlap PCR is commonly used as a method for mutagenesis [19], but it was used in this experiment as a method for combining two PCR products in a direction manner, as seen in Diagram 3. Single-extension overlap PCR differs from regular PCR in that it isn't used mainly as a DNA amplification method. In this experiment it was

used primarily to combine two DNA fragments and then to amplify the product. The PCR procedure used to make the initial fragments to be used in single-extension overlap PCR was modified slightly. The PCR reaction for what would be the transit peptide had a primer with an extension beyond that of the DNA of interest hanging off the 3' end. The other piece had a complementary section hanging off the 5' end. In the following PCR reaction, the two segments are bound together and the polymerase makes a copy that is covalently bound.

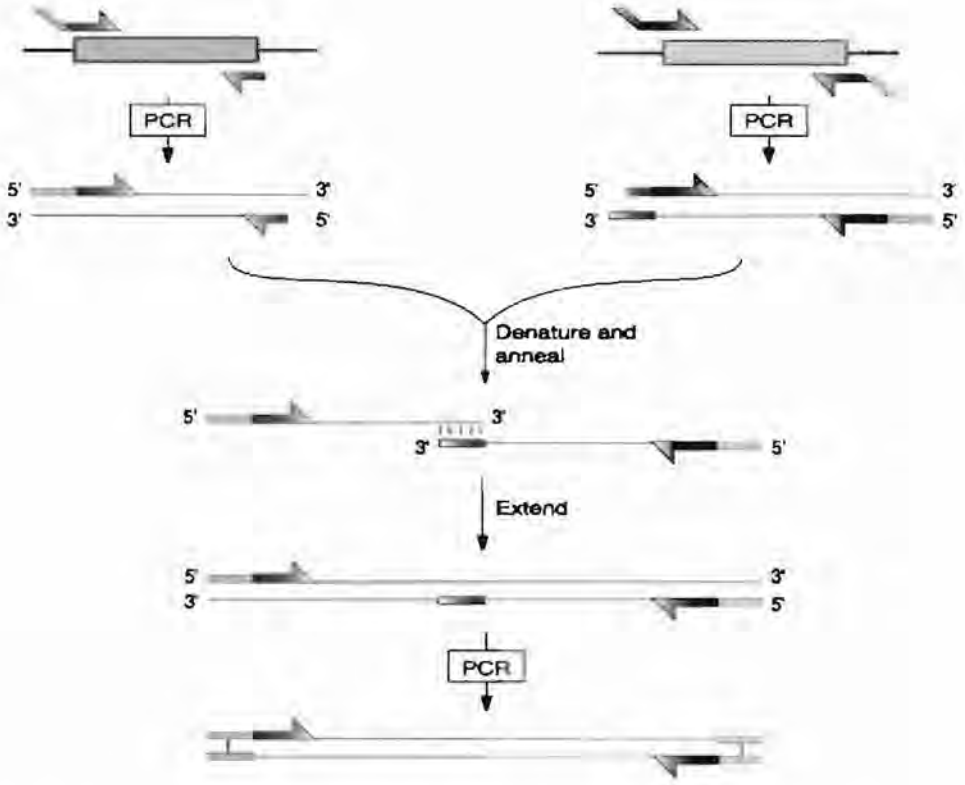

Diagram 3: Single-extension overlap PCR takes two different PCR products, each designed with a single stranded overlap. The first overlap is complementary to tbe second, as seen in the diagram. After the first complete desired DNA fragment is obtained, PCR continues as normal. This diagram was modified from [11].

This procedure allowed for the easy attachment of two different PCR products, giving the needed end product.

Experimental

The first step of my research was to obtain colonies of *E. coli* containing a plasmid that had the acyl carrier gene. A stock of this strain was available in deep freeze from Dr. Katherine Schmid, but growth medium had to be made. The medium used was Luria-Bertani solid medium (LB) which contained 10.0 g of bacto-peptone (DIFCO Laboratories), 5.0 g of yeast extract (DIFCO Laboratories), and 10.0 g of sodium chloride (NaCl) (VWR International). The bacto-peptone provides amino acids, the yeast extract supplies vitamins and other essential nutrients, and the sodium chloride is used mostly for osmoticum. Agar (DIFCO Laboratories) was added to the liquid medium to allow it to gel. The agar was dissolved and the medium was sterilized in the autoclave (Getinge Castle). To ensure that the bacteria grown on the plates would be those that contained the desired plasmid, sterile ampicillin (Sigma) was added to the medium before it was poured using sterile methodology into plates at a final concentration of 100 μg/mL medium. After the plates had hardened, the *E. coli* was inoculated onto them with a flame-sterilized inoculating loop. The plates were then incubated at 37° C overnight.

Liquid LB was made as above except without the addition of agar and 3 mL of it was aliquoted per 18 mm diameter test tubes. These were sterilized in the autoclave. Once they had cooled, single colonies from the plates were picked with sterile toothpicks and dropped into separate LB containing test tubes. The tubes were then incubated at 37° C in an incubator/shaker (New Brunswick Scientific) overnight at 200 rpms. The next day the plasmid DNA was isolated from the bacteria by the alkaline lysis mini-prep method was used on the cultured bacteria [10]. From each test tube, enough LB with cells was removed to fill a labeled 1.5 mL microfuge tube. The microfuge tubes were spun in a microcentrifuge at

13,000 rpm for one minute to separate the cells and the LB which was full of cellular metabolic waste. As much of the LB supernatant as possible was then removed. 100 μL of the ice cold glucose reagent was added to the cells which were then resuspended by pipetting up and down. The glucose reagent contained 0.9% glucose, 2% 0.5M ethylene diamine tetra acetic acid (EDTA), and 1.25% Tris pH = 8. Afterwards, 200 μL of alkali reagent (4.7 mL H_2O, 96 μL 10 M NaOH, and 240 μL 20% SDS) were added and the tube was inverted multiple times to mix. The tubes were then allowed to stand for five minutes, no more or less. Otherwise, the DNA yield would have been very small. After exactly five minutes, 150 μL of the acetate reagent (20 mL 5M potassium acetate, 11.4 mL H_2O, and 4.6 mL glacial acetic acid) was added and the tubes were vortexed for ten seconds. The tubes were then placed on ice for a minimum of five minutes and were then placed into the microfuge for a five minute spin at 13,000 rpm.

The supernatant was removed from the pellet which consisted of micells and protein. In a hood, 450 μL of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the supernatant and the tubes were vortexed briefly. They were then microfuged at 13,000 rpm for three minutes. The supernatants, now containing plasmid of interest and other molecules of similar characteristics and little else, were removed to fresh tubes and had 1 mL of 95% ethanol added to each. Each tube was inverted multiple times to mix and then spun in the microfuge at 13,000 rpm for twenty minutes. This step was done to precipitate the DNA. The ethanol was then taken off carefully to avoid disturbing the DNA pellet. 500 μL of 70% ethanol were then added to dilute the remaining contaminants in the pellet. The tubes were then microfuged again for ten minutes at 13,000 rpm. The ethanol was poured off, once again being careful to avoid disturbing the pellet. Next, the tube was lightly flicked to get as

much ethanol off the pellet as possible. The excess ethanol in the tube was then removed with a sterile Q-tip. The pellet was left to dry for fifteen minutes. The DNA was then resuspended in TE8 (10 mM Tris, 1 mM EDTA, pH 8).

To determine if the plasmid obtained by the miniprep was indeed the one of interest, each sample was cut with the restriction enzyme EcoRI (NEB). The digestion with EcoRI was run at 37° C for one hour. Each reaction contained 1 μL of the appropriate buffer (buffer H), 2 μL of DNA, 6.5 μL of water, and 0.5 μL of EcoRI. The cut plasmid preparations were run on an agarose gel with 0.089M Tris, 0.089M boric acid, 1 mM EDTA (TBE) as shown in Figure 1. Gel electrophoresis is used to separate linear DNA based on its size and can be used as an effective analytical tool.

Figure 1: Lanes 1 through 12 are different trials of the same miniprep which was used to determine the presence of the acyl carrier gene in pBluescript. The two positive control lanes are a known sample of the plasmid cut with the same restriction enzymes as the other samples. The other lane is the same known plasmid that was not digested. The bands indicating the plasmid are boxed in red.

The minipreps that appeared the best were used to continue the work. These steps were repeated with bacteria containing the CFAS gene in a plasmid.

The next step was to perform the polymerase chain reaction (PCR) on the plasmids to obtain an abundance of the transit peptide and the CFAS gene. However, unlike normal PCR, the primers I used had a specific single-stranded sequence hanging off the 3' end for

the transit peptide. There were actually two versions of the reverse primer for the transit peptide, with second primer being slightly longer than the first. When a transit peptide led polypeptide is taken into a plastid, the transit peptide is cleaved. The first primer ends at the cleavage site for the transit peptide, however, the nucleotides after the cleavage site determine whether cleavage will happen or not. Therefore, a second primer was also used, which contains fifteen addition bases (or five amino acids worth) after the cleavage site, which are found in spinach and known to work. The CFAS gene had a 5' single stranded sequence. The 5' extension off of the CFAS gene and the 3' extension off of the transit peptide were complementary. The PCR reactions were set up as shown in Table 1 and Table 2. The annealing temperature used in all PCR reactions for this experiment was 72° C. The PCR primers (supplied by Sigma Genosys) used are as follows:

Transit peptide forward primer: 5'-CTAAGCTTATGGCTTCCATCACTGGAT-3'

Transit peptide reverse primer 1: 5'- GCAAGAGACACTTAGGCCAC-3'

Transit peptide reverse primer 1: 5'- CTCGGGCTTGGCAG-3'

CFAS forward primer 5'- CTAAGCTTATGAGTTCATCGTGTATAGAAG-3'

CFAS reverse primer 5'- CTGAATTCTTAGCGAGCCACTCGAAGG-3'

The underlined sequence in the transit peptide forward primer is the HindIII restriction site, and the underlined section of the CFAS reverse primer is an EcoRI restriction site. The PCR products for the transit peptide were run on an agarose gel (Figure 2) and the bands were cut out using a sterile scalpel on a UV-light box.

Figure 2: This gel contains the PCR products obtained for the transit peptide from the acyl carrier gene. The two reverse primers used cause 3' single stranded extensions. Lanes 1 through 5 contain PCR of the DNA from lane 1 from Figure 1. Lane 1 is a PCR product using just the forward primer. Lanes 2 and 3 contain the reverse primer without the forward primer. Lanes 2 and 3 each use a different reverse primer for the transit peptide, which gives different lengths of single stranded overlaps. Lanes 4 and 5 are the complete PCR of the transit peptide using the reverse primers from lanes 2 and 3 respectively. The bands are boxed in red for clarity. Lane 6 contains the DNA without the primers and lane 7 contains the primers without the DNA.

DNA template (1µL)	10X PCR buffer	forward primer	reverse primer 1	reverse primer 2	dNTPs	water	Taq polymerase
Acyl Carrier Protein	5 µL	5 µL			5 µL	34.5 µL	0.5 µL
Acyl Carrier Protein	5 µL		5 µL		5 µL	34.5 µL	0.5 µL
Acyl Carrier Protein	5 µL			5 µL	5 µL	34.5 µL	0.5 µL
Acyl Carrier Protein	5 µL	5 µL	5 µL		5 µL	29.5 µL	0.5 µL
Acyl Carrier Protein	5 µL	5 µL		5 µL	5 µL	29.5 µL	0.5 µL
Acyl Carrier Protein	5 µL	5 µL			5 µL	29.5 µL	0.5 µL
	5 µL	5 µL	5 µL		5 µL	33.5 µL	0.5 µL

Table 1: The PCR reaction mixture recipes to obtain the transit peptide with corresponding primers to obtain 3' extensions. From top to bottom the reaction recipes are lanes 1-7 on Figure 2.

The portions cut from the gel were weighed and the DNA was extracted using a Qiagen extraction kit following the manufacturer's instructions. A sample of the extracted transit peptide was run on another gel (Figure 3).

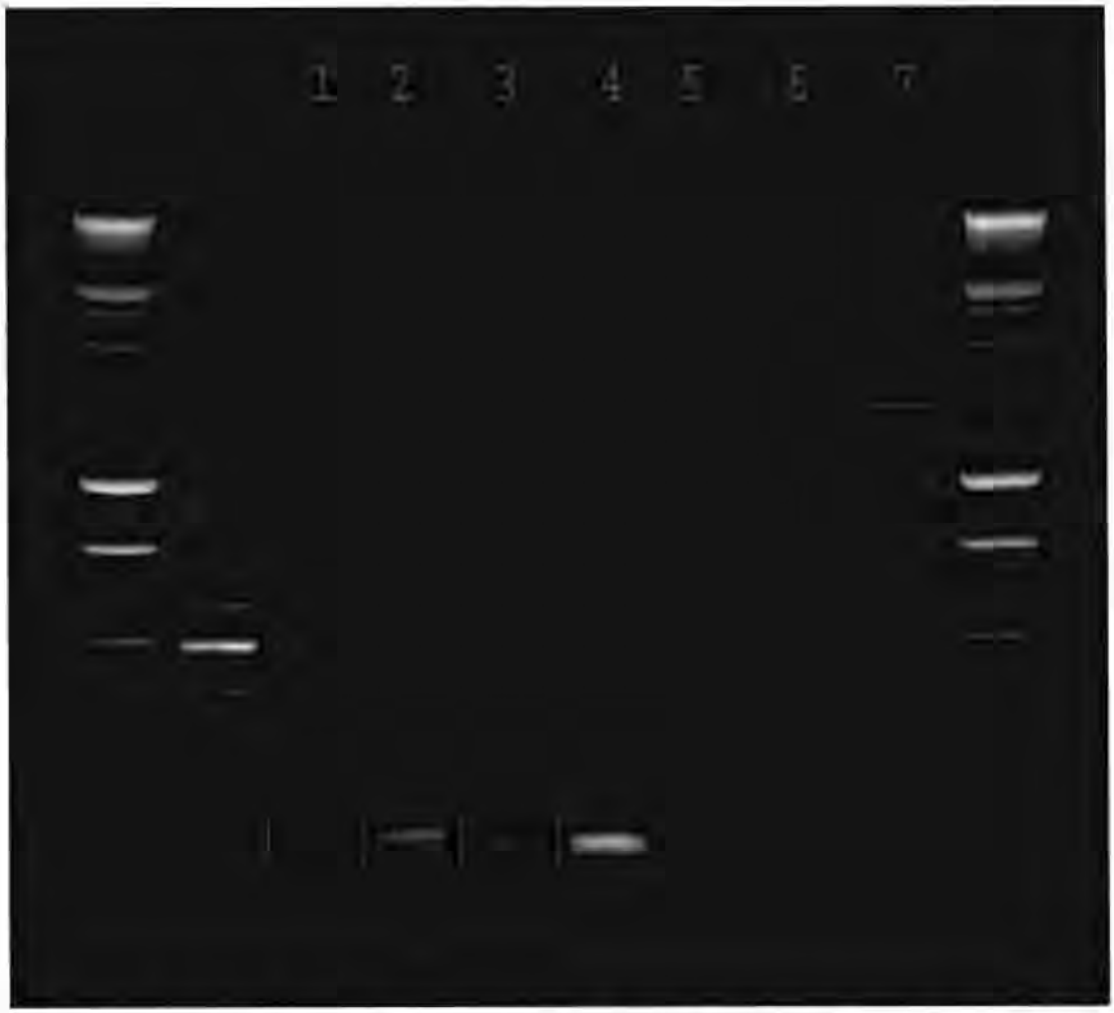

Figure 3: This gel shows the products of the elution of the DNA from the previous gel. Lane 1 shows the eluted product from lane 5 on Figure 2, which is shown again in lane 2. Lane 3 is the eluted product from lane 4 of Figure 2, which is shown in lane 4. Lanes 5 through 7 aren't important to the project. The boxed lanes are the two eluted products.

Figure 4 shows the PCR reaction for the CFAS gene (top) and another set for the transit peptide (bottom).

Figure 4: Lanes 1 through 5 contain PCR products of the L12 plasmid which contains the CFAS gene. Lane 1 contains a forward primer that gives a single stranded overlap that is complementary to the reverse overlaps of the transit peptide. Lane 2 shows a positive control with the CFAS gene with normal primers giving no overlap. The primers were known to work well. Lane 3 is similar to lane 1 except the PCR was missing the reverse primer. Lane 4's PCR was missing the forward primer. Lane 5's PCR reaction used the normal forward primer but no reverse primer. Lanes 6 through 11 show the transit peptide from acyl carrier gene with controls. It shows the same information as Figure 2. The boxed bands are the CFAS gene (top) and transit peptides (bottom).

DNA template (1µL)	10X PCR buffer	forward primer	reverse primer 1	dNTPs	water	Taq polymerase
CFAS	5 µL	5 µL	5 µL	5 µL	28.5 µL	0.5 µL
CFAS	5 µL	5 µL (control)	5 µL	5 µL	28.5 µL	0.5 µL
CFAS	5 µL	5 µL		5 µL	33.5 µL	0.5 µL
CFAS	5 µL		5 µL	5 µL	33.5 µL	0.5 µL
CFAS	5 µL	5 µL		5 µL	33.5 µL	0.5 µL

Table 2: The PCR reaction mixture recipes to obtain the gene for CFAS with corresponding primers to obtain 5' extenstions matching the 3' extensions of the transit peptide. The control primer is a primer that contains no overlap. The reaction recipes from top to bottom match lanes 1 through 5 of Figure 4.

The DNA eluted from the red highlighted CFAS bands of Figure 4 were eluted from the gel and an aliquot was run on another gel (Figure 5) to ensure that the elution was successful. Once again, the transit peptides are also shown eluted from the gel in Figure 4.

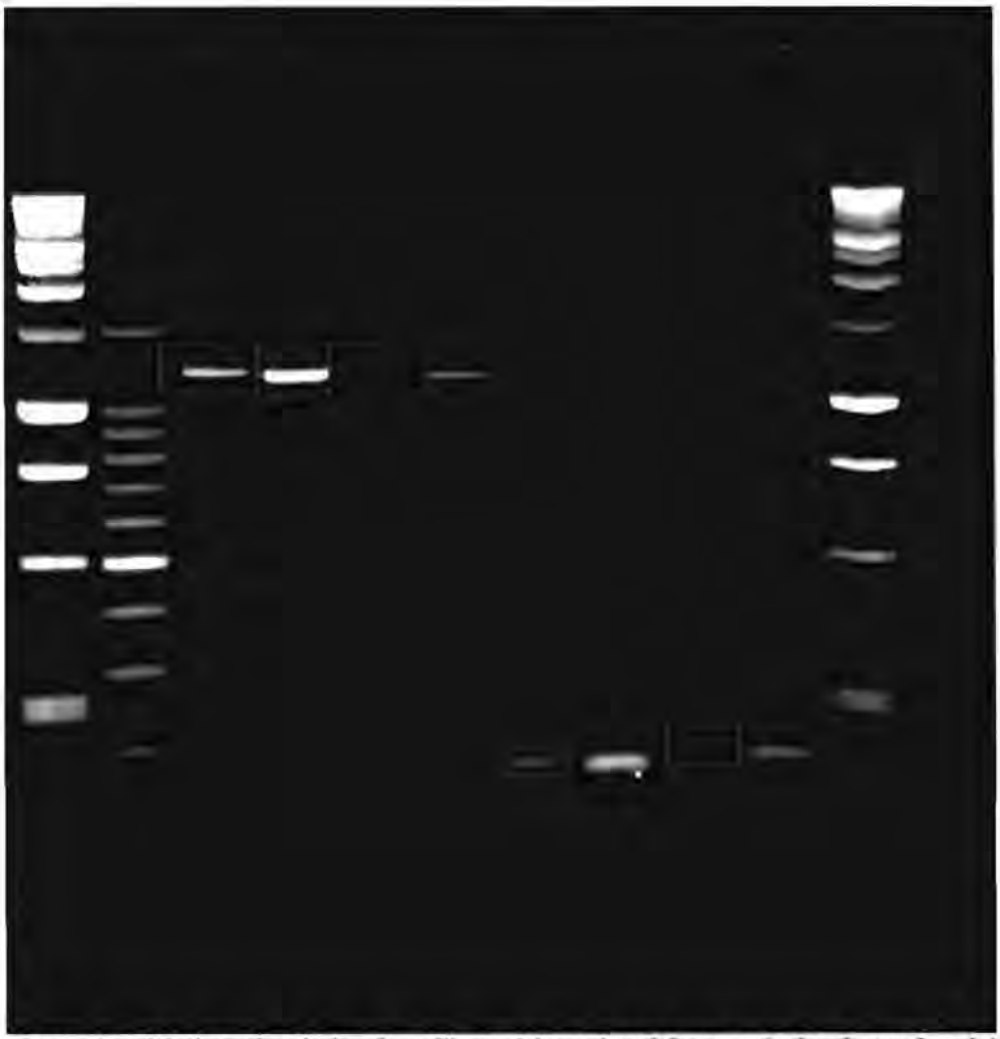

Figure 5: Lanes 1 and 3 show the elution from Figure 4 lanes 1 and 2 respectively. Lanes 2 and 4 represent those lanes. Lanes 5 and 7 show the elution from Figure 4 lanes 6 and 7 respectively, which are shown in lanes 6 and 8. The boxed bands are the gel purified bands.

Afterwards, single-extension overlap PCR was performed using both the transit peptide and the CFAS gene, as shown in Table 3. The PCR products were run on a gel (Figure 6) and eluted as before. The eluted DNA was run on another gel to ensure that the DNA was successfully removed from the gel.

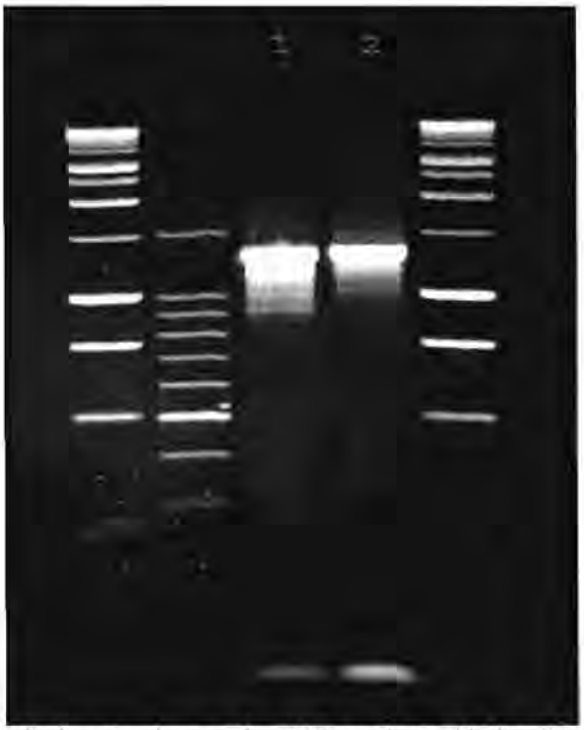

Figure 6: Lane 1 shows the single-extension overlap PCR product with the CFAS gene with forward overlap and the transit peptide with the shorter reverse overlap. Lane 2 shows the same thing except that the transit peptide has the longer reverse overlap.

DNA 1 (1 µL)	DNA 2 (1 µL)	10X PCR buffer	forward primer (T-peptide)	reverse primer (CFAS)	dNTPs	water	Taq polymerase
CFAS	T-peptide Reverse Overlap 1	5 µL	5 µL	5 µL	5 µL	27.5 µL	0.5 µL
CFAS	T-peptide Reverse Overlap 2	5 µL	5 µL	5 µL	5 µL	27.5 µL	0.5 µL

Table 3: The single-extension overlap PCR reaction recipes.

Next, the *E. coli* containing the plasmid pGA748 and *E. coli* containing pGA748 with the CFAS gene were pulled out of storage and grown on LB plates. The cells were put through the mini-prep procedure previously described. The plasmid obtained was digested with restriction enzymes to ensure it was indeed pGA748 and that the second set did have the

CFAS gene in it. Next, some of the cells remaining in the test tubes were used to inoculate 1 L of LB medium to perform a maxi-prep, which is just a large scale version of the mini-prep. A Qiagen kit was used and the manufacturer's directions were followed. The steps mirror the mini-prep, but the resulting DNA tends to be much more pure due to being obtained through ion-exchange chromatography. pGA748 is a low yield plasmid, meaning that there is a very limited number of copies possible in a bacterial cell at a given time. That is the reason a maxiprep was performed rather than a miniprep, in addition to the increased purity level. The DNA obtained was digested with EcoRI and run on the gel shown in Figure 7.

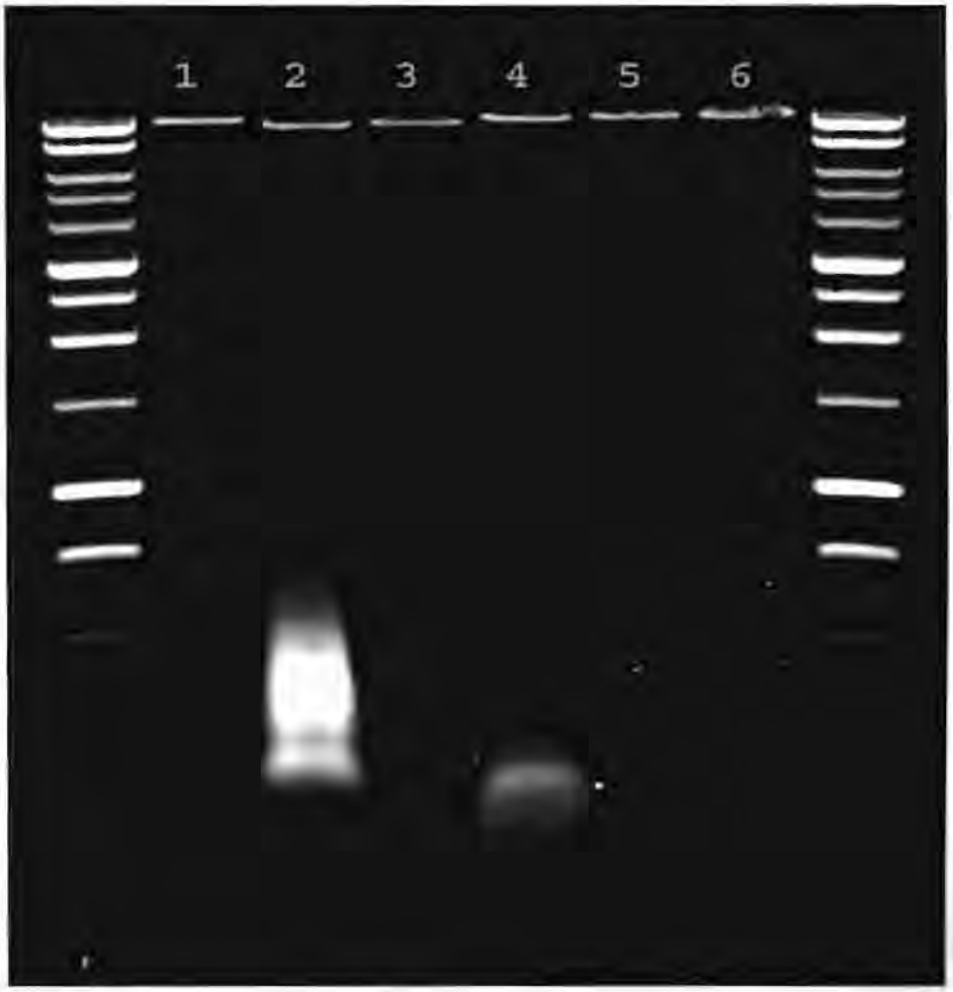

Figure 7: Lanes 1 through 3 have pGA748 and lanes 4 through 6 contain pGA748 with the CFAS gene.

The DNA obtained was viewed using UV spectroscopy at a dilution of 100 fold to determine its relative purity and concentration using Beer's Law. Figures 8 and 9 show the absorbance data obtained. Figure 8 shows the data for pGA748 and Figure 9 shows the data for the pGA748 containing the CFAS gene.

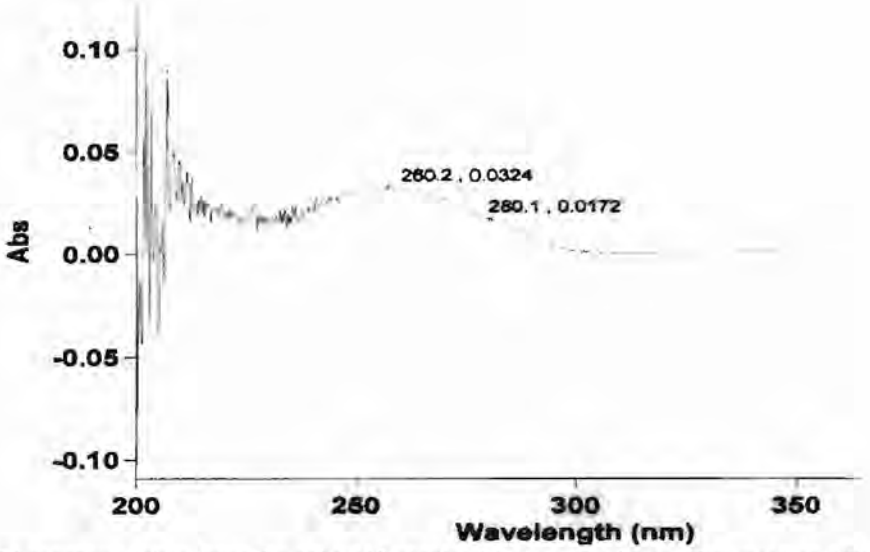

Figure 8: The UV spectrum taken from the pGA748 obtained from a maxiprep. The peak at 260 nm of 0.0324 can be used to get the concentration of the DNA and the ratio of the scan between 260 nm and 280 nm gives a general idea of the purity.

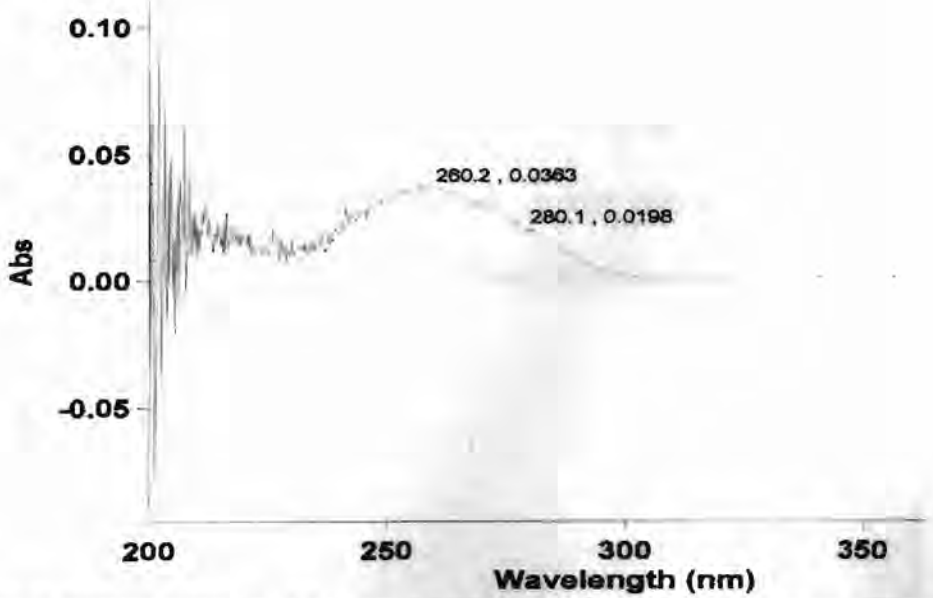

Figure 9: The UV spectrum taken from CFAS containing pGA748 obtained from a maxiprep. The peak at 260 nm of 0.0363 can be used to give the concentration of the DNA.

Then the plasmids obtained from the maxi-prep, as well as the CFAS gene were digested with EcoRI and HindIII and run on a gel (Figure 10).

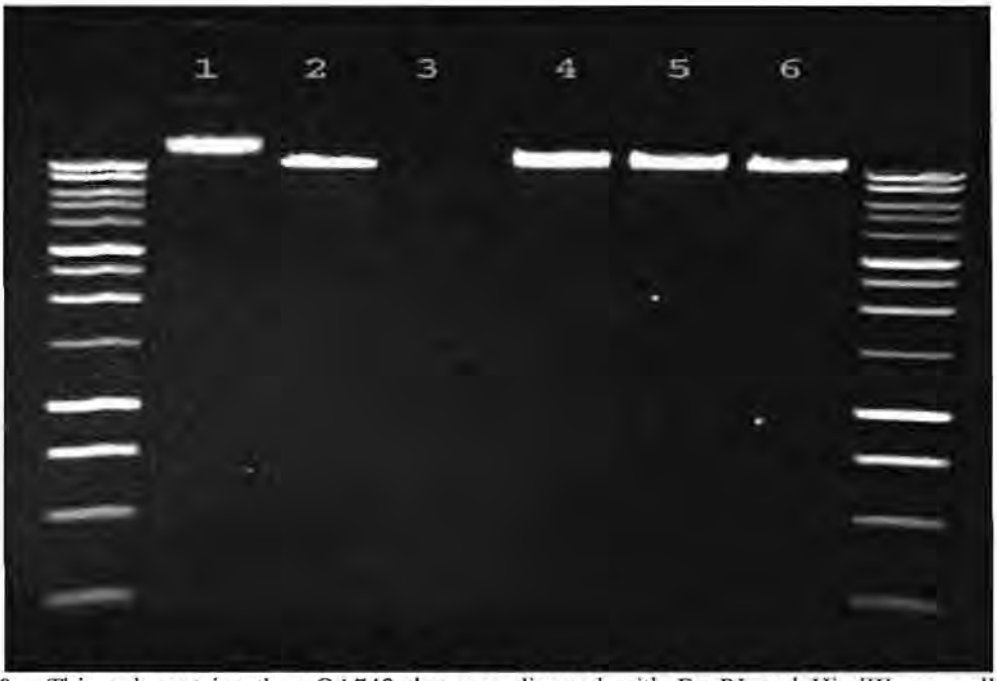

Figure 10: This gel contains the pGA748 that was digested with EcoRI and HindIII, as well as being dephosphorylated. This is shown in lanes 4, 5, and 6. There are three identical lanes because a large amount of plasmid was treated with the enzymes. Lanes 1 and 2 show CFAS containing pGA748 digested with EcoRI and HindIII. The CFAS segment is not visible.

These specific enzymes allow for directional cloning, as described previously. pGA748 was also treated with calf intestinal alkaline phosphatase to ensure that the ligase would not close the plasmid on itself, but only with the recombinant CFAS gene. The dephosphorylation was done during the restriction digest because the phosphatase works well in restriction buffers and it reduces the need for phenol and chloroform treatments. Both the plasmid and the recombinant CFAS gene were then gel purified.

Discussion

All of the bands in Figure 1 are wavy and not very clear. This could be because the restriction digest wasn't run correctly, or the EcoRI used wasn't very reliable, although the same stock was used later with more success. The smear at the bottom of each set of lanes is RNA left over from the miniprep. Because RNA is very chemically similar to DNA, it is also isolated during the procedure. RNase was added to all minipreps following this to reduce this problem. Each miniprep was successful in obtaining the correct plasmid, but some preparations were better than others. The quality of the extraction is not exceedingly important because PCR can work on very few copies of DNA. DNA from lane 1 was used for the rest of the experiments.

Figure 2 shows the products of a PCR reaction to obtain the transit peptide apart from the plasmid it was contained in. The transit peptide obtained has a single stranded overlap extending off of the 3' end. There are two variations on the overlap primer used just in case one happened to not work so well. Lanes 1 through 3 contain the DNA from partial PCR reactions that were missing one of the primers. The bands are much longer, as is expected. The nature of PCR requires two primers to obtain a specific and discrete segment of DNA. Using only one primer gives the polymerase no boundaries on how far to go, so the segments are hundreds of base pairs longer and worthless. Even so, these bands do serve as a control exactly because they behaved just as expected. The other lanes contain negative controls, which show little with no strong bands, as expected. The DNA sample for lanes 4 and 5 in Figure 2 contains a lot of enzyme and PCR buffer, as well as unused primer and the original template DNA. To remove all of this contamination, just the desired bands are cut from the gel and purified out of it. This leaves the sample containing just the DNA, some left over

agarose, and the buffer used to dissolve the agarose. To insure that the DNA was successfully obtained from the gel, a sample of it is run on another gel next to the original PCR products, which is shown in Figure 3. As expected, the gel purified bands are the same size as the corresponding PCR bands.

This set of experiments was run again next to a new set. The new set of experiments consists of obtaining the CFAS gene with a 5' single stranded overlap. The single stranded overlaps on the CFAS gene and the transit peptide are complementary to one another so that they will hydrogen bond together and thus link the two pieces. The two segments are not yet covalently bound, therefore single-extension overlap PCR was performed. Single-extension overlap PCR works on the same principles as normal PCR. The same enzyme, buffer, and primers are used. The only difference is that the template DNA is not one continuous segment because the backbone of the CFAS gene and the transit peptide are not joined. However, the backbones are still right next to each other because of the complementation of the overlaps. Being held together by hydrogen bonding, the polymerase makes a copy of the whole transit peptide/CFAS DNA segment. The copy is one complete recombinant gene. The product of the reaction is the connection of the transit peptide with the CFAS gene.

The bands in Figure 7 all show the plasmid pGA748, a Ti plasmid for *Agrobacterium*. Ti plasmids from *Agrobacterium* tend to be much larger than typical *E. coli* plasmids, which is why the bands are at the very top of the gel. pGA748 is approximately 12,000 base pairs in length. A sample of pGA748 containing the CFAS gene already was obtained as well. This is important for use at the very end of the experiment to give a control to compare the transit peptide/CFAS recombinant gene to after transformation of an actual plant. The fuzziness at the bottom of the gel is just left over RNA from the miniprep.

The maxiprep that was performed on pGA748 and pGA748 with CFAS was quantified with UV spectroscopy. The UV spectrum seen in Figure 8 is fairly typical of pure DNA as seen by the ratio between 260 nm and 280 nm at approximately 1.8. Figure 9 is the UV spectrum from the pGA748 with CFAS and it has a ratio of almost exactly 1.8 between 260 nm and 280 nm. The amount of the two plasmids obtained and their purity was sufficient to continue the work with.

The pGA748 was digested with EcoRI and HindIII. In the same reaction mixture, it was also treated with calf intestinal alkaline phosphatase in order to dephosphorylate the open ends after restriction digestion. The transit peptide/CFAS recombinant gene was also digested with EcoRI and HindIII, but it was not dephosphorylated, because that would make ligation impossible with the pGA748.

What needs to be done now is to actually perform the ligation of the pGA748 and the recombinant CFAS gene. Then, the plasmid needs to be transformed into *E. coli* and many copies need to be made and isolated. Afterwards, *Agrobacterium* would be transformed with the recombinant CFAS containing plasmid and the CFAS containing plasmid. Tobacco cells would then be transformed using the *Agrobacterium*. The cells would have GC/MS performed on them to determine if the enzyme was functional in the plastids. The two different transformations would be looked at side-by-side to see if adding the transit peptide and sending the enzyme to the plastid was more effective than just sending CFAS to the cytosol.

That's the end goal of the project, but if there was not a difference between the two strains a couple of other pathways could be followed. A different transit peptide could be used to see if the source of the transit peptide caused differences in the way the gene was

moved into the plastid. Plastids have multiple membranes in them, which is a cause for concern because the enzyme could go to the wrong place in the plastid itself. Another possible method would be to use a different CFAS gene. The one used in this experiment is from bacteria, not from a plant. Having a true eukaryotic g ene would likely be more effective than a prokaryotic gene.

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