Tangerine Blush:

Engineering soybean to produce β-Carotene in seed cotyledons

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A. Objectives

The ultimate goal is the creation of soybean with a value-added trait of production of canthaxanthin. However, because multiple genes are required for canthaxanthin production, the immediate goal is to engineer soybean for production of β-carotene, a precursor for canthaxanthin, in its seeds.

Cell lines will be established and used for the genetic engineering process; engineered plants will be regenerated from these cell lines, and the resulting plants analyzed for proper gene expression.

B. Abstract

Consumers associate quality of food with color and flavor. Without certain carotenoids egg yolks, butter, and even shrimp turn white instead of their expected color. Hence, carotenoids must be incorporated into animal diets to obtain products that meet consumer expectations. Such carotenoids can be one of the most expensive feed ingredients. Soybean meal is a chief source of animal feed in the US, but is devoid of carotenoids. If soybean feed already contained carotenoids, farming industries would have a more economical choice for animal feed. The goal of the project was to genetically engineer soybean to produce carotene, the precursor for other agriculturally important carotenoids. Soybean embryos were shot in a gene gun with an engineered plasmid containing the phytoene synthase (crtB) from *Erwinia uredovora* gene for β-

carotene production, placed behind a cotyledon specific promoter and with hygromycin resistance as the selectable marker. Forty engineered lines were obtained. One line of the forty has turned orange denoting the successful expression of carotene during differentiation in the seed tissues, and two have turned orange during the embryo stage.

C. Key Words

carotene pathway, soybean transformation, microprojectile bombardment, β-carotene

D. Introduction

Soybean primarily becomes animal feed for the farming industry. Many of these industries, such as shrimp farms as well as egg producers, spend money on both feed and coloring agents (Guerin et al. 2003). Because the animals in farms cannot go out and eat different foods, they often do not have the natural coloring that people associate with them. To appeal to different markets and quality
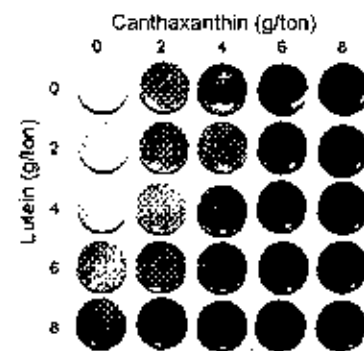
Figure 1: Colors of chicken egg yolks. The color of the yolk changes depending on amount of canthaxanthin and lutein consumed. Picture courtesy of Dan Fletcher, Poultry Science, The University of Georgia.

standards, farmers must feed coloring, usually carotenoids, to their animals to achieve different colors in their products (Figure 1). This means feed that already possesses coloring nutrients presents a more economic choice.

Carotenoids are synthesized in plants, bacteria, and algae. In plants, carotenoids act as accessory pigments during light-harvesting (Demmig-Adams et al. 1996), anti-oxidants (El-Agamey et al. 2004) and color components for fruits and flowers. β-carotene specifically has provitamin A activity (Maynes 1996) and is converted into

retinal, vitamin A, in human intestines (Lindqvist and Andersson 2002).

β-carotene has already been introduced into rice by Ye et al. (2000) to produce 'Golden Rice'. From this we know that it is possible to insert genes into plants to get carotenoids in tissues that normally would not produce them. Soybean has the precursor for phytoene, geranylgeranyl diphosphate and the enzymes to convert phytoene into β-carotene (Figure 2). β-carotene, orange in color, serves as a precursor for the production of zeaxanthin (yellow), or canthaxanthin (pink). The target goal, cathaxanthin, is a coloring that has not been cost-effectively reproduced in laboratories, and is usually the most expensive feed component.
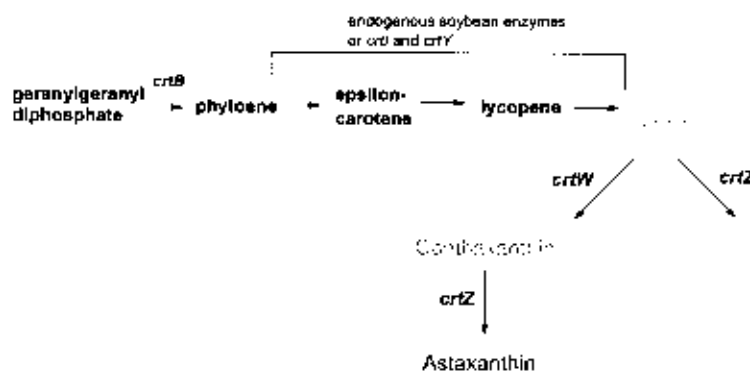

Figure 2: A diagram of the carotenoid pathway in soybean.

To take a normal colorless strain of soybean and modify it to produce an orange, yellow, pink, or our target red color, requires at least two steps. This project is focused on the first step in the expression of canthaxanthin in soybean seeds: converting phytoene and lycopene to β-carotene by introducing a gene obtained from *Erwinia caratova*. The trick is to associate the production of β-carotene in the seed, rather than any other part of the plant.

Creating a type of soybean that expresses β-carotene in its seed requires two processes: first the gene must be engineered, and then the gene must be introduced to the

soybean, which then must be raised and tested for the presence and variance of
expression of the target gene (Figure 3). Now that the gene has been created, the second
step must be accomplished. Physically introducing the engineered gene to the soybean
embryos requires the use of the gene gun (Hazel et al. 1998). This instrument shoots gold
dust that has been bound to purified DNA into soybean embryos; the embryos are then
taken and grown on selective medium until they are old enough to test for presence and
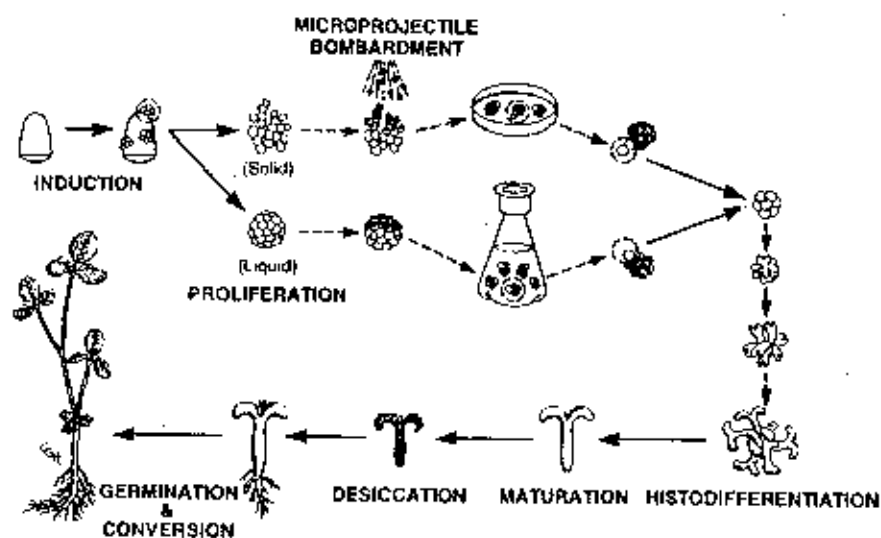

Figure 3: An overview of the transformation and embryo recovery process.

expression of the intended genotype and, ultimately, phenotype.

E. Materials and Methods

Media

Name	Abbreviation	Ingredients per Liter	Reference
Murashige-Skoog	MSD20	100 ml MS Macro stock 1 ml MS Micro stock 10 ml Ca stock 10 ml Fe stock 2 ml B₅ Vitamins stock 100 ml 2, 4-D stock 30 g Sucrose	Wright et al. 1991

		brought to pH 5.8	
	MSD40	100 ml MS Macro stock 1 ml MS Micro stock 10 ml Ca stock 10 ml Fe stock 2 ml B, Vitamins stock 200 ml 2, 4-D stock 30 g Sucrose 2 g Gelrite brought to pH 7.0	Finer & Nagasawa 1988 Modified from: Komatsuda & Ohyama 1988 Lazzeri et al. 1987
Finer-Nagasawa-lite with 200 µl of Hygromycin stock	FNLiteH20	100 ml FN-lite Macro stock 1 ml MS Micro stock 10 ml Fe stock 6.82 ml Ca stock 2 ml B, Vitamins stock 1 g Asparagine 25 ml 2, 4-D stock 10 g Sucrose brought to pH 5.8 200 µl of Hygromycin stock	Samoylov et al. 1998 Modified from: Finer & Nagasawa 1998 Chu et al. 1975
Soybean Histodifferentiation and Maturation	SHAM	100 ml FN-lite Macro stock 1 ml MS Micro stock 10 ml Fe stock 6.82 ml Ca stock 2 ml B5 Vitamins stock 0.149 g Methionine 30 g Sucrose 30 g Sorbitol brought to pH 5.8 110 ml Glutamine stock	Modified from: Samoylov et al 1998 Finer & Nagasawa 1988 Chu et al. 1990 Walker & Parrott 2000 Dyer et al. 1987 Skokut et al. 1982
	MSOlite	100 ml MS Macro stock 1 ml MS Micro stock 10 ml Ca stock 10 ml Fe stock 2 ml B5 vitamin stock 15 g Sucrose 2 g Gelrite brought to pH 5.8	Murashige and Skoog 1965 Gamborg et al. 1968

Stock Solutions

Name	Ingredients per Liter	References
2, 4-Dichlorophenoxyacetic Acid	0.200 g 2, 4-D	
B₅ Vitamins	5.00 g Thiamine·HCl 0.50 g Nicotinic Acid 0.50 g Pyridoxine·HCl 50.0 g Myo-Inositol	Gamborg et al 1968
Ca stock	44.0 g $CaCl_2·2H_2O$	

Fe stock	3.720 g Na_2 EDTA·$2H_2O$ 2.780 g $FeSO_4$·$7H_2O$	
FN-lite Macro Stock	4.63 g $(NH_4)_2SO_4$ 28.30 g KNO_3 3.70 g $MgSO_4$·$7H_2O$ 1.85 g KH_2PO_4	Samoylov et al. 1998
Hygromycn stock	100 mg mL^{-1} hygromycin	
MS Macro stock	16.5 g NH_4NO_3 19.0 g KNO_3 3.70 g $MgSO_4$·$7H_2O$ 1.70 g KH_2PO_4	Murashige & Skoog 1962
MS Micro stock	6.2000 g H_3BO_3 16.900 g $MnSO_4$·H_2O 8.6000 g $ZnSO_4$·$7H_2O$ 0.2500 g Na_2MoO_4·$2H_2O$ 0.2500 g $CuSO_4$·$5H_2O$ 0.8300 g KI	Murashige & Skoog 1962

Culturing from cotyledons and then embryos

Immature seed pods were collected from a stock of "Jack" soybean held in our greenhouse. The pods were cleaned and then sanitized using first a bath of 70% isopropanol for thirty seconds. Then 20% bleach is poured onto the pods and left to sit for 20 minutes. Afterwards the pods are washed three times with sterile water, and then left to sit in the water for 10 minutes to wash away any bleach remaining. Each pod is then split open in an sterile glass Petri dish, and each immature seed is removed from the pod. The seed's two cotyledons are separated and placed onto a MSD40 plate with what used to be the inside of the seed, or the flat part of the cotyledons, facing up from the medium plate. These cotyledons were placed into 5 columns with 5 rows to make plates of 25 cotyledon halves. Once finished, the plate is sealed with Nescofilm made by the Alfresa Pharma Corporation in Osaka, Japan in the hood and then moved to the culture room. This room is kept at a constant 24°C, 1-6 $\mu E\ m^{-2}\ s^{-1}$ of light, with a photoperiod of 16 hours of light and 8 hours of dark.

After six weeks, the cotyledons produce soybean embryos. The best embryos will be green and perfectly round, signaling that they have not started to differentiate. These embryos are then transferred, in the same five by five patterns, to MSD20 plates. Every month the new green embryos must be maintained by transferring them from old MSD20 plates to new MSD20 plates and returned to the culture room.

Four days before shooting a plasmid, embryos must be taken off their old MSD20 plates and put onto new medium. Instead of arranging the embryos in their usual five by five patterns, the embryos to be transformed are placed into a tight 2.5 cm diameter circle in the center of the plate. After four days on new medium the transferred embryos are at their peak of growth and highly transformable (Hazel et al. 1998).

Plasmid

The soybean embryos were transformed with the plasmid pCEH1B. pCEH1 is a hygromycin selection vector cassette containing a seed-specific promoter and a chloroplast transit peptide sequence designed to produce carotenoid pathway genes in seed plastids (Figure 4). Addition of a chloroplast transit peptide (TP) sequence of a pea *rbcS* gene to a seed-specific promoter (soybean lectin, *GleP*) was done using an overlap PCR technique to create pGle10_TP. Plasmid pGle10_TP was digested with *Xba*I and ligated
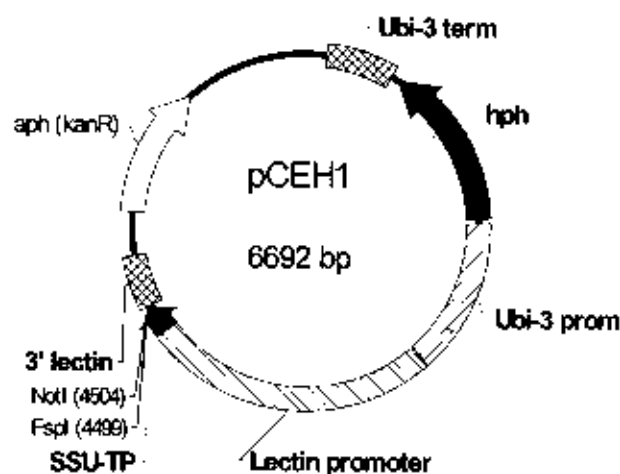

Figure 4: The plasmid pCEH1 used to create pCEH1B. The gene *crtB* was inserted in the *Not*I and *Fsp*I sites.

with *Spe*I-linearized pUHU-UGA creating pLPTPUHU. pLPTPUHU was digested with *Pmel/EcoRI*, treated with T4 DNA polymerase and ligated with linearized pSMART-HC Kan vector (Lucigen) creating pCEH1B. The *Fsp*I and *Not*I sites are used to insert a gene sequence as a translational fusion with the pea TP.

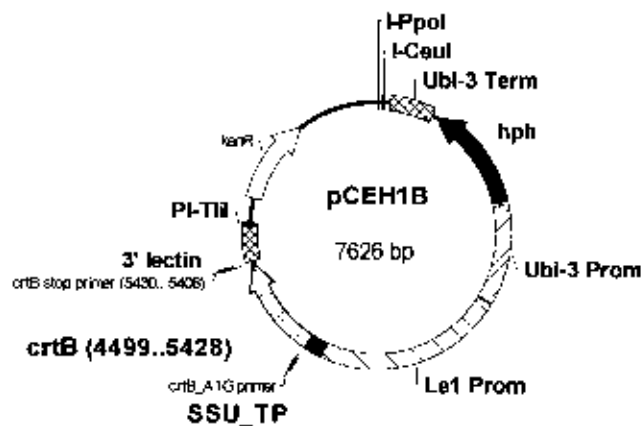The *crtB* gene was PCR-amplified with 35 cycles at 60°C from *Erwinia uredovora* genomic DNA (ATCC 19321D) using crtB_ATG and crtB stop as primers and cloned into pBluescript (blunted *Xba*I site) creating pcrtB. *crtB* was PCR-amplified from pcrtB with crtB_ATG and T7 primers, digested with *Not*I and ligated with *Fsp*I/*Not*I-linearized pCEH1 creating pCEH1B (Figure 5). Pfu polymerase (Stratagene) was used for PCR.

Figure 5: A map of the pCEH1B plasmid used to transform the soybean embryos.

Plasmid purification Bio-Rad

The Bio-Rad plasmid mini-prep kit was used on a culture of *E. coli* containing the pCEH1B plasmid. First, the cells were pelleted by centrifuging them at 14,000 x g for 30 seconds. Then, 200 μl of Cell Resuspension Solution was added along with 250 μl of Cell Lysis Solution. The tube was gently inverted until the solution was viscous. Next 250 μl of the Neutralization Solution was added and the tube was inverted until a precipitate formed. The tube was centrifuged at 14,000 x g for 5 minutes. The supernatant was removed and transferred to a spin filter with 200 μl of suspended matrix.

The spin filter was then centrifuged for 30 seconds at 14,000 x g. The filtrate was discarded, and 500 μl of Wash Buffer was added to the spin filter. The spin filter was centrifuged again for 30 seconds, the filtrate was discarded, and the Wash Buffer process was repeated once again. Finally, the spin filter was moved to another tube and 100 μl of DI water were added. The tube was centrifuged at 14,000 x g for one minute. The filtrate now contains the purified plasmid.

Binding plasmids to gold in preparation for shooting

Transformation was carried out using metal particle/helium bombardment in the gene gun. First the macrocarriers, Bio-Rad Cat. No. 165-2335, and the 7584 kPa rupture disks, Bio-Rad Cat. NO. 165-2329, were placed in a sterile plastic Petri dish filled with 95% EtOH in a hood. The stop screens, Bio-Rad Cat. No. 165-23336, were put into glass Petri dishes and sterilized in the autoclave. Then 10 mg of 0.6-micrometer gold dust was weighed out in a 1.5 ml tube. To this tube 1 ml of ethanol was added, and then it was sonicated and placed on ice for 30 second. The sonication and ice treatments were repeated twice more, and then the tubes were spun for 5 minutes at 7,000 rpm, and the ethanol was removed leaving behind the metal pellet.

Then 169 μl of EtOH was added to the tube and vortexed for 1 minute at setting 3. The suspension was then sonicated for 10 seconds twice. Thirty-five μl of the solution were transferred into separate 1.5 ml tubes. Each aliquot is good for 3 shots, or 3 plates. All of the aliquots were centrifuged at 10,000 rpm for about 10 seconds, and the supernatant was removed.

One ml of water was added to each aliquot and spun for 5 minutes at 2,000 rpm.

The water was removed and then 25 µl of DNA at 10 ng per µl concentration were added, and the pellet of metal was resuspended by pipetting up and down. The aliquots were then spun for 3 seconds and sonicated for 15 seconds.

Two hundred and twenty µl of Type I sterilized water were put into each aliquot and again resuspend by pipetting up and down. Then they were vortexed for 3 seconds and sonicated for 15 seconds. Two hundred and fifty µl of ice-cold 2.5 M CaCl were added, and then the tubes were vortexed for 3 seconds and sonicated for another 15 seconds. Finally, 50 µl of ice cold 0.1 M spermidine stock, which should be no older than four months, were added, and again the tubes were vortexed for 3 seconds and sonicated for 15 seconds.

The aliquots were capped and placed on ice for 3 minutes and then vortexed at setting 3 for 10 minutes. Next, they were centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and then the pellet was resuspended with 600 µl of 100% ethanol. This suspension was vortexed for 3 seconds and sonicated for 10 seconds.

Lastly, the aliquots were spun for 5 minutes at 1000 rpm and the EtOH was removed. The pellet was resuspended in 36 µl of 100% EtOH by flicking and sonicating for 7 seconds. The aliquots were left on ice for an hour before shooting them in the gene gun.

Microprojectile bombardment: Using the gene gun

The gene gun was used in a sterile hood. First, the helium tank was opened and the vacuum pump was turned on. Next, 10 µl of the bound plasmid solution was spread evenly onto each of the macrocarriers. The EtOH was allowed to evaporate, and the

macrocarrier was inserted into a macrocarrier holder before use in the gene gun. For each shot, the rupture disk retaining cap was removed, a rupture disk was placed into the retaining cap, and then the cap replaced on the gas acceleration tube and screwed in until tight. A stopping screen was placd onto the stopping screen support and a macrocarrier was inverted and placed on top of the fixed nest of the launch assembly with the microcarriers facing the stopping screen. The macrocarrier cover lid was screwed down and the assembly was inserted into the second shelf of the gene gun. The plate of embryos was opened and placed facing up into the gene gun 13 cm away from the macrocarrier assembly.

The gene gun was closed and a vacuum from the pump was applied. The fire button was pressed when the vacuum reached 91 kPa (27 in) of Hg. When the helium pressure exceeds the rupture disk's resistance, the gene gun fired. Immediately the fire button was released and the chamber was vented. The plate was removed from the gene gun chamber as well as the spent macrocarrier and rupture disk. The process was repeated for each of the plates. The shot plates of embryos were then sealed with Nescofilm and placed in the dark culture room for a week. The photoperiod in the dark culture room was 16 hours of light and 8 hours of dark. The light intensity in the dark culture room was 1-6 $\mu E\ m^{-2}\ s^{-1}$ and the temperature was a constant 24°C.

Selection of embryos

Each cluster of embryos on a plate was split into halves in a sterilized hood. Each half was placed into a 125 ml autoclaved baffled Erlenmeyer flask in the hood. Each baffled flask was filled with 25 ml of autoclaved FNLH20 liquid media cooled to room

temperature and put into a dark culture room on rotating plates set at 135 rpm. Each week the FNLH20 medium in the flasks was poured out into a beaker in the hood and replaced with fresh FNLH20 liquid medium. This process was repeated for eight weeks.

The end of selection and DNA extraction

After eight weeks in selection on FNL medium, each flask was poured into a sterile Petri dish in a sterile hood. The dish of embryos was placed under a sterilized microscope to look for embryos that still had a green tint to them. Each fully green embryo that survived selection was transferred to individual 125 ml autoclaved baffled Erlenmeyer flasks with 25 ml of FNLH20 in the hood. Less green embryos that had not died yet were transferred to MSD20 plates with 250 μl of hygromycin L^{-1} of medium on the off chance that they would survive.

Left to grow for about three weeks, the embryos on liquid medium were then poured out onto a plate in the hood and separated from dead tissue and other individual forming embryos. Small pieces of the embryos, about an eighth or as small as could be cut, were collected in 0.5 ml tubes for DNA extraction. The separated surviving embryos were poured back into original flasks and new FNLH20 was added.

DNA extractions for PCR screening was conducted on the collected embryos. They were first ground in 50 μl of CTAB buffer, and then 150 μl of CTAB were added after grinding was finished. The samples were then vortexed and placed into a 65°C water bath for 10 minutes. After the bath, 200 μl of phenol, chloroform, isoamyl alcohol (24:24:1) were added to each sample in the fume hood. The samples were then inverted about 20 times and centrifuged at 8000 rpm for 10 minutes. After the spin, the aqueous

layer on the top was removed and put into another tube. Two hundred μl of chloroform were added to the samples and then they were spun again for 10 minutes. Again the aqueous layer was removed and placed into another labeled tube. To these tubes 500 μl of chloroform, isoamyl alcohol (24:1) was added. The tubes were then inverted 20 times again and spun for 10 minutes at 8,000 rpm. The aqueous layer was removed and added to another new tube. Then 0.7 volume of isopropanol was added and then they were inverted gently as the DNA begun to precipitate. The DNA was pelleted by spinning for 10 minutes at 12,000 rpm. The alcohol was poured off leaving the pellet of DNA in the bottom of the tube. The pellet was washed by adding 100 μl of 70% ethanol and was then put through a short spin. The EtOH was then removed.

Polymerase chain reaction (PCR) testing

First, the extracted DNA was diluted to a concentration of 40 μg ml^{-1}. To test for the presence of the *crtB* gene, a PCR master mix containing 10.44 μl of deionized water, 3.2 μl of 5x GoTaq buffer with loading dye, 0.48 μl of each primer crtB_ATG and crtB stop, 0.32 μl of 10mM dNTPs, and 0.08 Go Taq polymerase per reaction was created. For all the 0.2 ml PCR tubes, 15 μl of the master mix was added. Then 1 μl of the 40 μg ml^{-1} sample DNA was added to an individual tube bringing the total volume to 16 μl. After the addition of the DNA, the tube was closed and labeled. Three controls were set up: two negative controls and one positive control. The first negative control was 1 μl of deionized water added to 15 μl of the master mix. The second negative control was 1 μl of extracted DNA from normal soybean added to 15 μl of the master mix. The positive control was 1 μl of 10 pg μl^{-1} pCEH1B plasmid dilution added to 15 μl of the master mix.

The samples and controls were run together in a thermocycler for 35 cycles with a middle step of 60°C for 45 seconds.

To test for the Hygromycin resistance gene *hph*, the same PCR master mix (Appendix) was created as before replacing the crtB_ATG and crtB stop primers with Hyg 117F and Hyg 938R primers. The same protocol for the *crtB* test described above was followed.

To test for the presence of the *GapC* gene, the same master mix (Appendix) was created as above replacing the two primers with GapC 59S and GapC 656R. The thermocycler was set for 35 cycles with a middle step of 55°C for 45 seconds.

After the thermocycler finished the 35 cycles, an agarose gel was made up by mixing 40 ml of 0.5x TBE buffer and 0.4 g of agarose. The solution was heated and 2 μl of ethidium bromide was added just before pouring the gel into a mold. The appropriate comb was placed into the liquid gel and the entire mold was left to cure. An electrophoresis tank was filled with 0.5x TBE buffer and the solidified gel was placed inside. Next, each of the samples from PCR was loaded into the wells of the gel next to 4 μl of a 100 kb ladder. The tank was set to run under 100 volts. When the gel had run far enough for the bands in the ladder to separate, it was placed on an ultraviolet light and a picture of the gel was taken.

Getting plants from embryo lines that test positive

When the individual embryos in FNL at the end of selection multiplied, each line that tested positive was poured into a sterile Petri dish in the hood. The best embryos, the greenest and roundest, were put back into their baffled Erlenmeyer flask with 25 ml of

new FNLH20 liquid medium. Several embryos were placed into another sterile Petri dish. These embryos were cut into small pieces with a sterilized scalpel, dipped in ethanol and then flamed in a Bunsen burner. This mass of sliced embryos was put into a new autoclaved baffled Erlenmeyer flask with 25 ml of SHAM liquid medium. The FNLH20 and SHAM flasks were placed back into the culture room on the original shake plates. The remainders of the embryos, left over from the FNL and SHAM flasks, were put onto back up MSD20H20 solid medium plates in case of contamination.

The SHAM flasks were checked at the end of every week for differentiation. After the embryos in the SHAM elongated and grew cotyledons, they were removed from their SHAM media and put into sterile Petri dishes in the hood. The Petri dishes were sealed up with a small square of MSOlite solid media inside. These Petri dishes were returned to the dark culture room for a week of desiccation.

After being desiccated for a week, the dried soybean embryos were moved, trying to orient the cotyledons pointing up out of the media, from the Petri dishes to plates of MSOlite solid media. These plates were labeled and put into the light culture room set at 26 °C, 66-95 μE m^{-2} s^{-1}, with a photoperiod of 23 hours of light and 1 hour of dark. As the soybean plants grew roots and leaves, they were transferred to boxes with MSOlite medium to avoid cramping them in small plates.

Each soybean plant was then transferred to an autoclaved box containing soil and water to grow by itself. These were left for about 2 weeks until the plants were taken to the greenhouse and replanted into larger buckets in non-sterile conditions.

Southern Blot Analysis of PCR positive plants

First, DNA from leaf or embryo samples was extracted.

Extraction and Thin Layer Chromatography of Carotenoids

Samples for carotenoid extractions were frozen on liquid nitrogen and then freeze dried. They were then ground using a mortar and pestle. The extraction process used was described by Fraser et al. (2000). The entire extraction was done in a dark hood as far away from strong direct light as possible. First, 100 µl of methanol was added to the samples which were then vortexed for 5 minutes. Next, 100 µl of Tris-HCL (50 mM, pH 7.5 containing 1 mM NaCl) were added to the samples, which were then immediately mixed and placed on ice for 10 minutes. The samples then had 400 µl of $CHCl_3$ added to them. They were immediately mixed and left on ice for another 10 minutes. The organic phase, or hypophase, was separated by centrifuging the samples at 3,000 g for 5 minutes at 4°C. The organic phase was removed and put into a marked 1.5 ml centrifuge tube. The aqueous phase of the sample was re-extracted by adding 400 µl of $CHCl_3$ and placing the sample on ice for 10 minutes again. The samples were centrifuged again for 5 minutes at 3,000 g and 4°C. The pooled organic phases were then dried on ice under nitrogen conditions. Then, 50 µl of CHCl3 was added to each dried extract so they could be applied to the silica gel plates.
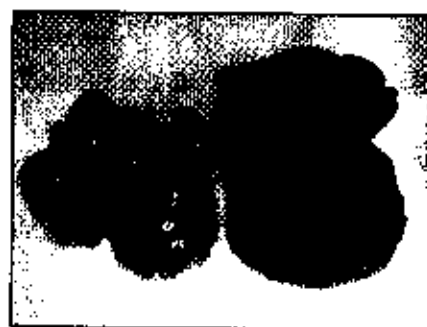
The thin layer chromatography

F. Results and Discussion

Twenty-five plates of embryos have been shot. Five plates were shot at a time on five different days: March 4, 2005; April 1, 2005; January 23, 2006; January 27, 2006; and March 3, 2006. Forty cell lines have been

Picture 1: PCR test of embryos for *crtB* run at 60°C for 35 cycles. The positive control (not shown here) showed a band, and the negative control (not shown here) did not.

recovered so far. Of these forty, eight have tested

positive for the *crtB* insert during the embryo stage:

R1, R3, B1 (discussed later), J2, J3, J4, W2, and W3

(Picture 1). Four cell lines did not test positive for

crtB as embryos but did survive the eight weeks of

hygromcin selection on FNLH20. Two cell lines, J3

Picture 2: An untransformed soybean embryo (left), and a transformed orange embryo expressing the *crtB* gene (right) from the J3 cell line.

and W2, have turned orange as embryos (Picture 2). One cell line, R3, turned orange as

it differentiated (Picture 3).

Picture 3: Embryos of the R3 cell line. Numbered from left to right: 1. and 2. two embryos after three weeks of SHAM media. 3. An embryo, R3, after a month on MSM6 media with charcoal. 4. A normal soybean embryo.

Two cell lines, R1 and B1, have

survived to become adult plants. Leaf

tissue was tested for the *crtB* gene and the

hph gene (Picture 4). Embryos from the

R3 cell line never grew roots while on

MSOlite medium, and so did not survive

to become plants. Four more cell lines are

undergoing histodifferentiation on SHAM

medium, and five more are expected to be put

on SHAM soon.

Seeds were taken from the adult R1 and

B1 plants. Twelve seeds from each cell line

were planted, and leaf tissue samples from ten

of the germinated seeds in each cell line were
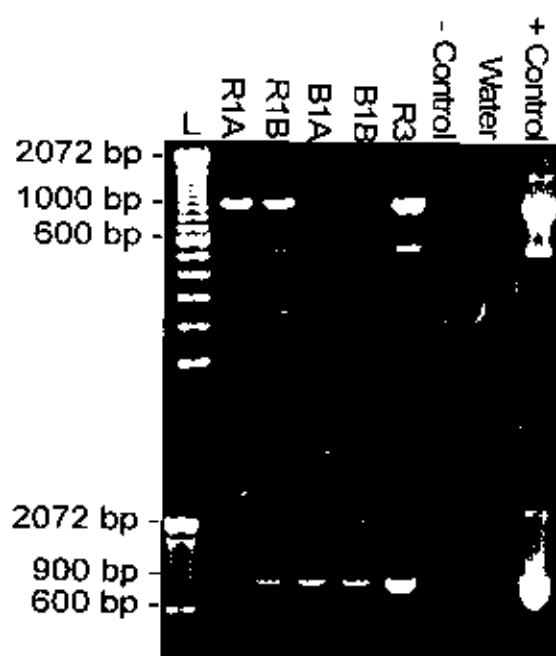
Picture 4: PCR testing of engineered leaf tissue. DNA extracted from non-engineered soybean was used for the negative control, and the positive control was 10 pg of the pCEH1B plasmid. The top half of the gel shows bands for presence of the *crtB* gene in the plant genome. Bands in the bottom half indicate the presence of the hygromycin resistance gene.

collected. None of the ten plants tested positive for the presence of *crtB* in the B1 cell line progeny. Three tested positive in the R1 progeny and seven tested negative (Picture 5). This does not fit the 3:1 ratio, and so twelve more seeds will be planted and ten more second generation plants will be tested for the *crtB* gene.

Southern blot analysis of PCR positive plants will also be done as final proof that the *crtB* gene was inserted. The cell lines that test positive after a Southern blot will be grown into adult plants, and their seeds will be collected for further testing. First, the seeds will be tested for the quantity of β-carotene produced in the seed. Next, the seeds will be planted to produce a second generation for testing inheritability of the *crtB* gene.

Picture 5: PCR testing of the R1 cell line progeny for the *crtB* gene run at 60°C for 35 cycles. The positive and negative controls are as described in Picture 4.

G. Conclusions

The project began with doubt that soybean could be engineered like "Golden Rice." The results suggest, however, that it is possible to redirect β-carotene expression to the seed, and thus provide the substrate needed to synthesize other carotenoids of value. Due to the successes demonstrated during this project, another project has started working towards our ultimate goal: targeting expression of canthaxanthin in soybean.

Works Cited

Chu, CC, CC Wang, CS Sun, H Chen, KC Yin, CY Chu, and FY Bi. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on nitrogen-sources. Scientia sinica 18: 659-668.

Demming-Adams B, AM Gilmore, WW Adams. 1996. Carotenoids 3: *In vivo* function of carotenoids in higher plants. FASEB J (10): 403-412.

El-Agamey A, GM Lowe, DJ McGarvey, A Mortensen, DM Phillip, TG Truscott, AJ Young. 2004. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. Arch Biochem Biophys 430: 37-48.

Finer, JJ, and A Nagasawa. 1988. Development of an embryogenic suspension-culture of soybean (glycine-max Merrill). Plant Cell Tissue and Organ Culture 15: 125-136.

Fraser, PD, MES Pinto, DE Holloway, and PM Bramley. 2000. Application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. The Plant Journal 24: 551- FIND LAST PAGE

Gamborg, OL, RA Miller, and K Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Experimental cell research 50: 151-158.

Guerin M, ME Huntley, M Olaizola. *Haematococcus* astaxanthin: applications for human health nutrition. Trends Biotechnol, 2003, 21:210-216.

Hazel, CB, TM Klein, M Anis, HD Wilde, and WA Parrott. 1998. Growth characteristics and transformability of soybean embryogenic cultures. Plant Cell Reports 17: 765-772.

Komatsuda, T, and K Ohyama. 1988. Genotypes of high competence for somatic embryogenesis and plant-regeneration in soybean Glycine-max. Theoretical and Applied Genetics 75: 695-700.

Lazzeri, PA, DF Hildebrand, and GB Collins. 1987. Soybean somatic embryogenesis – effects of nutritional, physical and chemical factors. Plant Cell Tissue and Organ Culture 10: 209-220.

Lindqvist A, and S Andersson. 2002. Biochemical properties of purified recombinant human β-carotene 15, 15'-monooxygenase. Journal of Biological Chemistry 277: 23942-23948.

Mayne ST. 1996. β-carotene, carotenoids, and disease prevention in humans. FASEB J (10): 690-701.

Murashige, T, and F Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Railey, L, EMA Enfissi, N Misawa, W Schuch, PM Bramley, and PD Fraser. 2004. Metabolic engineering of ketocarotenoid formation in higher plants. The Plant Journal 39: 477-486.

Samoylov, VM, DM Tucker, F Thibaud-Nissen, and WA Parrott. 1998. A liquid medium-based protocol for rapid regeneration from embryogenic soybean cultures. Plant Cell Reports 18: 49-54.

Samoylov, VM, DM Tucker, and WA Parrott. 1998. Soybean [Glycine max (L). Merrill] embryogenic cultures: the role of sucrose and total nitrogen content on proliferation. In Vitro Cellular & Developmental Biology-Plant 34: 8-13.

Tan J, N Baisakh, N Oliva, V Parkhi, M Rai, L Torrizo, K Datta, SK Datta. The

screening of rice germplasm, including those transgenic rice lines which accumulate beta-carotene in their polished seeds, for their carotenoid profile. International Journal of Food Science and Technology, 2005, Vol 40, Iss 5, pp 563-569.

Wright, MS, KL Launis, R Novitzky, JH Ducsing, and CT Harms. 1991. A simple method for the recovery of multiple fertile plants from individual somatic embryos of soybean [Gliycine max (L.) Merrill] induced by cocultivated *Pseudomonas* maltophilia. In Vitro Cellular & Developmental Biology-Plant 27: 153-157.

Ye, X., S. Al-Babili, and A. Klöti. 2000. Engineering the provitamin A (b-carotene) biosynthetic pathway into (carotenoid free) rice endosperm. Science 287: 303-305.

Appendix:

Formulas:

CTAB (200ml)

4g CTAB

8ml 0.5 EDTA

0.18g ascorbic acid

20ml 1M Tris

56ml 5M NaCl

4g PVP-40

137mg DIECA

800ul B-Mercaptoethanol

Bring to volume with water

PCR Master Mix

(per reaction)

10.44 µl H_2O

3.2 µl 5x buffer/loading dye

0.48 µl primer 1

0.48 µl primer 2

0.32 µl 10mM dNTPs

0.08 µl Go Taq Polymerase