Using *Brassica oleracea* To Produce A Cure For Malaria

A Major Qualifying Project

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ABSTRACT:
Artemisinin is an antimalarial compound found in *Artemisia annua* at about .01-.1% DW. This in combination therapy is the most potent treatment we have against the parasite *Plasmodium falciparum,* curing even cerebral malaria.

Brassica oleracea (Kale) has a large amount of carotenoids, a 40 carbon molecule, whose basis is 10 carbon monoterpenes. In this project we used *A. tumefaciens* to insert genes into *Brassica oleracea* that code for enzymes that produce farnesyl diphosphate and amorpha 4, 11-diene as well as a cytochome P450 that catalyzes multiple oxidations of intermediates. Through this we hope to show that the production of the precursor for artemisinin in the form of artemisinic acid is possible in larger quantities than they occur in *A. annua*.

INTRODUCTION:

A sesquiterpene (a group consisting of $C_{15}H_{24}$) known as artemisinin is taken from the plant *Artemisia annua*. This chemical is used for anti-malarial treatment world wide. The most successful form of this treatment, is artemisinin-based combination therapies(ACTs)[1]. A second use that has been found is possibly the treatment of cancer. Artemisinin appears to be cytotoxic to cancer cells, having effects on cell proliferation and angiogenesis. One of the problems with this compound is that it occurs in extremely low quantities naturally, making up 0.01-1% of the plant's dry weight.

Artemisinin, a terpenoid, is an end result of complex series of reactions starting from the use of acetyl-coenzyme A (Acetyl CoA)[2]. Terpenoids are one of the largest families secondary metabolites found in plants. A secondary metabolite is a compound not directly used in photosynthesis or respiratory metabolism[3]. Monoterpenes are found mostly in plants. They are used for many things ranging from being used to create commercial flavors and scents[4]. Acetyl-CoAis formed in the metabolic breakdown of carbohydrates and fatty acids. Three acetyl-CoA molecules are condensed in a two step reaction to hydroxymethylglutanyl-CoA which is then reduced to mevalonic acid. Mevalonic acid then undergoes a two step phosphorylation consuming two ATPs to form a six carbon mevalonic acid pyrophosphate. A carboxyl group is then removed resulting in a five carbon compound, isopentyl pyrophosphate (IPP) and its isomers. The biosynthesis of terpenes uses one of each of these molecules and condenses them to form a 10 carbon unit, geranylpyrophosphate (GPP). Additional groups can be added in the same head to tail method to form increasingly larger groups. The 15 carbon intermediate formed by this is called farnesylpyrophosphate (FPP) and is the fundamental building

block for the formation of artemisinin. The formation of FPP occurs in the cytosol along with resulting triterpenes and further 15 carbon additions. A similar pathway also occurs in the plastid, but entirely avoids the mevalonic acid pathway. The plastidial pathway utilizes Pyruvate and Glyceraldehyde 3-phosphate as its starting compounds rather than Acetyl-CoA. The two pathways have been shown to have some amount of interaction[5,6] most notably with IPP. Farnesyl diphosphate synthase (FPS) is utilized in the conversion of IPP to FPP. FPP is converted into amorpha-4, 11-diene via catalysis by amorphadiene synthase (ADS)[7].

Cytochrome P450s are a large superfamily of proteins[8] responsible for the metabolizing of drugs, as well as pollutants, steroids and other compounds. CYP71AV1, is responsible for catalyzing the next few steps of the pathway, yielding artemisinic alcohol, then taken to artemisinic acid[1]. Artemisinin is thought to form in the glandular secretory trichomes (GSTs). These appear as tiny hairs, and can either secrete or contain a fluid, although artemisinin has been found in transformed roots as well[9]. Transformed roots are the result of DNA is recombining with host genome added via a vector such as *Agrobacterium rhizogenes*, and is then replicated along with the rest of the genome[14]. Plants that have been transformed with this bacteria have what is known as "hairy roots". Artemisinin has been shown to accumulate in highly specialized glandular tissues in these roots, and is not normally detectable in wild type plants[10]. mRNA has been extracted from GSTs found on leaves. This allows us to extract the cDNA and amplify it via reverse transcriptase-polymerase chain reaction (RT-PCR).

For years science has tried to find a way to synthesize artemisinin in large quantities. While scientists have been successful in chemically synthesizing artemisinin,

we still do not completely understand the complex method by which it is formed *in vivo*, and the yields are still low. There are some plants with a naturally high amount of carotenoids. These come from the fundamental building block of isopentyl pyrophosphate (IPP). This compound is a precursor for all terpenoids. What we endeavor to do is to genetically modify *Brassica oleracea (*kale) to produce artemisinic acid from the large amount of precursors (IPP), the plant naturally produces in the plastid.

Artemisinin has a long history of use to battle illness. *Artemisia annua* has been mentioned in Chinese writings as far back as 340 B.C. for treatments of fevers. In the middle of the last century the Chinese government, looking for a cure for malaria started reviewing these old texts. As a result they came across qinghaosu, widely known as artemisinin. Since its isolation, various other treatments for malaria have been found such as: chloroquine, doxycycline, sulfadoxine-pyrimethamine (Fansidar®), mefloquine (Lariam®), and atovaquone-proguanil (Malarone®)[11] Multi-strain drug resistance of *Plasmodium falciparum* is becoming far more of a problem in recent years[12]. One constant treatment that appears to work, even in the case of drug resistant strains is a combination therapies utilizing artemisinin[3].

There are a few proposed mechanisms of action for the effects of artemisinin on the malarial parasite *Plasmodium falciparum.* The first is that it interfs with a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). The SERCA is responsible for maintain calcium ion concentrations needed for things such as calcium mediated signaling and some post-translational protein modifications, as well as protein folding. It has been shown that this targeting by artemisinin is so specific that even at higher concentrations (50μM) it doesn't affect other transporters, this includes the non-SERCA

Ca^{2+}-ATPase PfATP4. Artemisinin is thought to bind in the same region as a known SERCA Ca^{2+}-ATPase inhibitor called thapsigargin (Tg). This was experimentally supported by application of both thapsigargin and artemisinin and observing a decrease in artemisinic activity. When the artemisinin binds to the protein using hydrophobic interactions it leaves the peroxide bridge exposed which is then reduced via a Fenton (fig 1) reaction leaving a reactive oxygen species (ROS) this results in enzymatic deactivation and the death of the parasite. It has been shown in vitro that artemesinin is sterically sensitive. When smaller sidechains are replaced there is a large decrease in effective activity. This seems to indicate that artemisinin is activated after binding to a highly specific site. This is supported by studies of a single amino acid mutation of PfATP6 in *X. laevis* oocytes. The change of Leu263 alters the electrostatic interactions and prevents the binding of artemisinin to the protein[13].

$$Fe^{2+} + H_2O_2 \text{ ----> } Fe^{3+} + .OH + OH^-$$

$$Fe^{3+} + H_2O_2 \text{ ----> } Fe^{2+} + .OOH + H^+$$

(fig 1). A Fenton reactions where two different species of radicals are produced.

Another possible effect is the activation of artemisinin by transition metals in the mitochondria, causing the production of reactive oxygen species. The creation of these oxygen species depolarizes the mitochondrial membrane and causes the loss of membrane potential negatively impacting the cell's ability to produce pyrimidine; a necessity for the production of RNA and DNA[14].

There are several methods of producing artemisinin. The way that most commercial artemisinin is produced is from plants grown in a field. The problems with

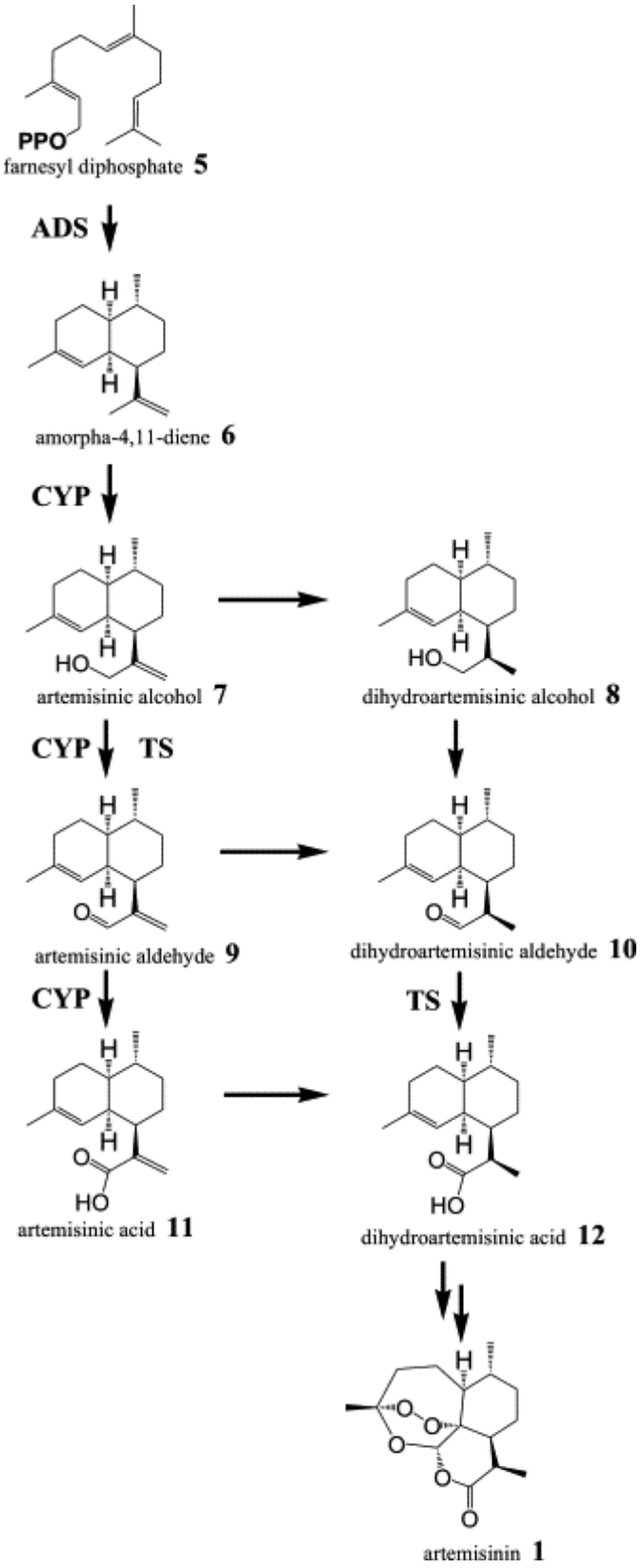
this are the same that all field grown plants face. There are droughts, fungi, bacterial and insect impacts, all of which could affect the outcome of the final product to be used medicinally. It has been shown that the possibility exists to synthesize artemisinin. It is a ten step stereo-selective process, but is costly and has very low yields. Artemisinin biosynthesis has been studied in different parts of the plant *A. annua* that biosynthesis of artemisinin was studied in, including callus, suspension cells, shoots, and hairy roots. Different techniques were utilized to influence artemisinin production, ranging from supplementation with hormones, to increased ratios of nitrate to ammonia. Other techniques compared looking at the difference in the synthesis from autoclaved media to filter sterilized media to alterations in the sugar that was used. Light and the wavelength of the light in addition to the temperature were found to be factors that contributed to artemisinin production as well[15].

It has been shown that overexpression of isopentyl transferase (*ipt)* elevates cytokinin levels in transgenic plants, and in this case, elevating artemisinin by 30-70% when compared to the control[15].

Another method for producing artemisinin was to use bacteria instead of plants. The model system used for this was *E. coli*. This research started by creating the mevalonate-dependant pathway in *E. coli.* Researchers enhanced the bacteria's natural production of IPP by taking all the enzymes necessary to synthesize FPP for the isoprenoid pathway from *S. cerevisiae* and expressing them in the *E. coli.* This was to take the large amounts of the precursor, acetyl-CoAand turn it into FPP. This pathway was coupled with an ADS pathway to produce amorpha-4, 11-diene. Artemsinin was not produced, however the researchers believe that the biochemical pathway could be

extended to produce artemisinic acid[16]. This has been achieved in engineered yeast in comperable amounts to *A. annua*, however growth occurs in a matter of days.

The Covello group, studying compartmentalization and regulation dealing with terpene biosynthesis have found that they could obtain a larger quantities of of they coupled the overexpression of taxadiene synthase with an inducible transcription system in Arabidopsis. In plants they demonstrated that larger amounts of terpenes are produced in the plastid stating that cytosolic production is highly regulated[4]. Terpenes are a highly volatile compound[2] and easily evaporate. Given that the cytosol is less contained that the plastid, we hypothesize that the difference in accumulation is not because the process is highly regulated in the cytosol, but escapes from the cytosol more easily. Below is a proposed method of some of the pathways for the theoretical biosynthesis of Artemisinin.


PPO
farnesyl diphosphate 5
ADS
amorpha-4,11-diene 6
CYP
artemisinic alcohol 7
dihydroartemisinic alcohol 8
CYP
TS
artemisinic aldehyde 9
dihydroartemisinic aldehyde 10
CYP
TS
artemisinic acid 11
dihydroartemisinic acid 12
artemisinin 1

MATERIALS AND METHODS:

DNA Mini-Prep:

A single bacterial colony was transferred into 2mL of LB medium containing 25µg/mL of kanamycin in a loosely capped 15mL tube. The culture was incubated overnight at 37^{o}C with vigorous shaking. After 24 hours 1.5mL of the culture was transferred into a microfuge tube and centrifuged for 30 seconds. The medium was removed leaving only the bacterial pellet, which was resuspended in 100µL of ice cold GTE solution by vigorous vortexing. 200µL of freshly prepared .2M NaOH and 1% SDS solution (on ice) were then added. The tube was mixed by rapid inversion 5 times to make sure the entirety of the contents came in contact with the solution. 150µL of ice cold 5MKOAc solution was then added and stored on ice for 5 min. The tube was centrifuged and the supernatant transferred into a new tube. 1 volume of phenol chloroform was added and again centrifuged for 5 minutes. The the layer was transfered to a new tube. The dsDNA was precipitated using 2 volumes of ethanol and put into the –80^{o}C freezer for 15 minutes. The mixture was allowed to stand at room temperature for 2 minutes before centrifuging for 15 minutes and removing the supernatant. The nucleic acids were redisolved in 30µL of TE (pH 8.0) containing 1µL RNAse and incubated at 37^{o}C for 30 min.

Preparation of Competent Cells:

2 tubes of 5mL ½YEP media containing 50µL/mL of rifampicin were prepared and inoculated from a glycerol stock of *Agrobacterium tumefaciens* and shaken at 28^{o}C. 24 hours later they were used to inoculate a culture of 500mL ½YEP again incubated

overnight with shaking. Cultures were checked 24 hours later OD600>.6. The cultures were transferred to the pre-chilled sterile 450mL centrifuge tubes and centrifuged at 2700 x g for 5min at 4^{o}C. From this point on all work was conducted at 4^{o}C. The supernatant was removed and discarded. The pellet was re-suspended in 100mL of pre-chilled glycerol (not vortexed). The suspension was centrifuged at 2700 x g for 5min at 4^{o}C. The supernatant was discarded and the pellet resuspended in 5mL of ice-cold 10% glycerol (not vortexed). The suspension was transferred and combined into new 35mL centrifuge tubes. The 450 mL tubes were rinsed twice with 5mL of 10% glycerol and this was also combined with the suspension. These were centrifuged at 2700 x g for 5 min at 4^{o}C. The supernatant was removed and the pellet was re-centrifuged for another minute to remove as much fluid as possible. A volume equal to the pellet of ice-cold 10% glycerol was added (about 1.5mL) and the pellet was re-suspended. The suspension was split in 40 μL aliquots and frozen overnight at –20^{o}C then transferred to –80^{o}C.

Electroporation:
DNA prepared in the mini-prep was put on ice as well as the electroporation cuvettes and the previously prepared competent cells. The competent cells were quickly hand thawed then put back on ice. 2 μL of miniprep DNA was put into a tube containing 20 μL of competent cells. They were mixed by tapping. The electroporator was set to a capacitance of 330, High Ω, charge rate of fast, and the voltage booster was set to 4kΩ. Ice was put into the chamber of the electroporator. DNA cell mix was put into the cuvettes without bubbles, and the apparatus was charged to 4V. After successful discharge the cells were incubated for 1-2 hours on LB media at 30^{o}C, after which it was spread evenly onto an

LB plate and incubated overnight at 28^{o}C. Transformants were selected for using kanamycin.

Medium Quick References:
All at pH 6.0

-MS media
- Murashige & Skoog salts

-Basal Medium
-MS medium, vitamins, 20g/L sucrose, 7g/L phytagel

-Shoot outgrowth Medium
-Basal Medium +40 mg/L adenine hemisulfate, 500mg/L polyvinylpyrrolidone, 1μg/L BAP

-Germination Medium
-MS medium set at 10g/L of sucrose, 7g/L phytagel

-Callus Inductin Medium
-Basal Medium, 3mg/L BAP, .5mg/L silver Nitrate, .2 mg/L NAA, .01mg/L GA_3

-Root Induction Medium
-Germination medium, .2gm/L IBA

-Shoot Induction Medium
-Same as Callus Induction Medium

-Transformant Selection Medium
Same as shoot outgrowth Medium, 10g/L sucrose.

Transformation:

Dwarf blue kale was obtained from a standard online gardening website. It was chosen specifically for its smaller size. Kale seeds were surfaced sterilized in bleach containing 6.5% sodium hypochlorite and shaken vigorously for 20 min. The seeds were then rinsed five times with sterile distilled water and placed onto germination medium. Cotyledons and hypocotyls sections were cut from four day old germinated seedlings and placed on

callus induction medium. The Hypocotyls and cotyledons were dipped in *A. tumefaciens* suspension OD650 = .05 containing pBTPADSTPFPS. After exposure to the bacteria for 1min the explants were immediately placed onto basal media with infected edge into the media, hypocotyls were placed vertically and co-cultivated with the bacteria for 2 days and then transferred to callus induction medium containing 500mg/L carbenicillin. From this point on all media contains 500mg/L of carbenicillin. 7 days later explants were transferred to shoot induction media containing carbenicillin and 25mg/L kanamycin. These explants were transferred to fresh medium every two weeks. When green shoots formed they were placed into shoot outgrowth medium containing carbenicillin and 25mg/L kanamycin. The shoots that remained green on this medium were then transferred to transformant selection medium containing 50mg/L. The shoots that remained green were finally transferred to root induction medium.

RNA extraction:

A leaf was cut off the kale and massed at .1g. This was frozen with liquid nitrogen, ground up and vortexed with .5mL cold PureLink^{TM} plant RNA reagent until resuspended. The tube was incubated horizontally at room temperature for 5min before centrifuging at 12,000g in a microcentrifuge for 2 minutes at room temperature. After transferring the supernatant to a new RNase-free tube .1mL of 5M NaCl was added to the clarified extract and the solution was mixed by tapping. .3mL of chloroform was added to the sample and mixed thoroughly by inverting the tube. The tube was again centrifuged at 12,000 x g for 20min at 4^{o}C, and the upper aqueous phase was transferred again to an RNase-free tube. To this there was an equal volume of isopropyl alcohol added. The solution was vortexed briefly and allowed to sit at room temperature for 10 minutes before being centrifuged at

12,000 x g for 20 minutes at 4°C. The supernatant was aspirated out carefully and 1mL of 75% ethanol was added to the pellet. The solution was centrifuged at 12,000 x g for 1 min at room temperature and the supernatant was again removed by aspiration. 20µL of RNase-free water was added to the RNA pellet and the pellet was resuspended and kept stored at -80°C.

Reverse transcription:

A mastermix was prepared using 4µL 10 buffer RT, 4 µL dNTP mix (5 mM of each dNTP), 4 µL Oligo-dT primer, 2 µL RNase inhibitor, 2 µL Omniscript Reverse Transcriptase and RNase free water to a final volume of 40 µL after the template would be added. The template RNA was added to individual tubes containing the master mix, vortexed briefly, centrifuged briefly to collect drops on the side of the tube and allowed to incubate at 37°C for 60 min.

BAP:

Dissolve in 1N NaOH and bring to volume with water.

Rifampicin:
Dissolve in methanol.

RESULTS:

Kale seeds were surface sterilized and germinated to produce hypocotyls and cotyledons for subsequent transformations. Seeds had a germination rate of 100% for the first five trials. 40 seeds were used for the first second and sixth trials, 30 were used in the third forth and fifth. The sixth trial using 40 seeds had a germination rate of 77% most likely

due to an overexposure to the sterilizing agent sodium hypochlorite. The transformation was performed with the cells containing *A. tumefaciens* containing the plasmid pBTPADSTPFPS for the first trial. The second and third trial utilized *A. tumefaciens* with pGGFPFAC2A The fourth and fifth trial utilized *A. rhizogenes* containing the plasmid pGGFPFAC2A. The sixth trial contains *A. rhizogenes* without a plasmid insertion. The first three trials had successful growths with an approximate transformation rate of 18% (on average 7 explants per trial survived on kanamycin media). For the first 4 trials hypocotyl lengths were 1cm. Better results were noted with smaller hypocotyls in the fifth and sixth trials, about a 15% transformation rate, so the hypocotyl size used was reduced to 3-5mm thus allowing 2 hypocotyl segments per seed to be used.

On the second and third attempts there were problems with overgrowth of bacteria. When the explants were plated on media containing carbenicillin there was still a large amount of bacterial growth. When this growth was noticed the explants were then rinsed in MS media and transferred to new media containing carbenicillin. The bacterial growth persisted and resulted in the explants turning white and dying. The company that produced the carbenicillin was contacted and we were informed it was a lower potency than their normal product, about 57% the expected potency.

A plant that was living on high kanamycin media from both the first and second trials were chosen and a leaf was taken from each (about 100mg of plant matter). The RNA was extracted using the method described in the materials and methods section. Using this RNA a reverse transcription was performed and the product was amplified in a PCR machine. The results of the PCR were negative for ADS expression.

Conclusions:

When first starting the series of experiments described in the earlier sections a hypocotyl length of approximately 1-1.5cm was used. Starting at the fourth trial due to smaller cotelydons and a lack to infect with bacteria smaller hypocotyl sections were utilized. It was found that these sections appear to have a better survival rate by about 25%. These numbers are approximate as since bacterial overgrowth interfered with the experiment.

The results of the transformations are still inconclusive. RT-PCRs were performed on leaves from plants from two different germination series, the first and the second. Both of these series show resistance to high kanamycin media, however the RT-PCR did not confirm the presence of our genes, specifically ADS. The first germination series was infected with *A. tumefaciens* containing a plasmid with transit peptides. These cells had the desired plasmid inserted along with a resistance to kanamycin to provide as a selective marker. The second germination series was infected using *A. tumefaciens* containing 2A sections of the foot and mouth disease virus under a single 35S promoter. The 2A section allows for ribosomal skipping and results in the uncatalyzed cleaving of proteins after being expressed in the ribosome. The polycistronic plasmid would mean that if ADS is expressed the entirety of the plasmid was being expressed at the same level. The third and fourth series were infected with *A. rhizogenes.* There was overgrowth on both of these series and they never survived to the point of being able to test for the presence of expression. The carbenicillin used during the third and fourth series was found to be of a lower potency (57%) than what it should have been. This and the difference in bacteria could have both been reasons for the overgrowth observed.

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