AliveFRESH: Engineering *Chlamydomonas reinhardtii* to produce geraniol

BIOSAFETY AND ETHICS

INNOVATION

VISUAL COMMUNICATIONS

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Climate change has been an ongoing global crisis and continues to worsen each year. In the last ten years, global temperatures have reached an all-time high. Additionally, burning fossil fuels and the emission of heat-trapping greenhouse gases have hastened climate change. These emissions include methane, nitrogen oxides, and carbon dioxide, which make up the greatest percentage of greenhouse gases. The most abundant greenhouse gas is carbon dioxide (CO_2). CO_2 has been produced by many factors related directly to humans, including the products of industrial factories and gas-powered vehicles. To combat this issue, AliveFRESH proposes a biological system that absorbs carbon dioxide while simultaneously producing a pleasant scent. Using a natural air freshener can avoid the negative impacts on human health, including the production of volatile organic compounds. The autotrophic single-celled organism, *Chlamydomonas reinhardtii,* would be engineered to produce geraniol, a monoterpene with a floral scent. *C. reinhardtii* was selected for this project because it is photosynthetic and uses a native methylerythritol phosphate (MEP) pathway. The light-independent reactions of photosynthesis will absorb carbon dioxide and produce G3P, a precursor to MEP. By utilizing the existing MEP pathway, AliveFRESH will produce isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which can convert into geranyl diphosphate (GPP) for subsequent monoterpene production. The final plasmid will include geraniol synthase (GES) and a red fluorescent protein (RFP) gene. With the addition of GES, GPP would convert into geraniol. RFP can be used to determine whether the transformation was successful by placing the culture of transformed algae under ultraviolet light. The AliveFRESH project creates a pleasant fragrance while also consuming carbon dioxide from the atmosphere. The information can be used to create a working plasmid through Addgene to be transformed in the future and is a potential solution to reduce the effects of climate change.

Keywords: *Chlamydomonas reinhardtii,* geraniol, carbon dioxide, MEP pathway

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Watch a video introduction by the authors at https://youtu.be/TXXAmNizd1k

Background

Affecting millions of people worldwide, the emission of greenhouse gasses from burning fossil fuels continues to have a negative effect on the environment and is the driving force behind climate change (United Nations, 2022). The results of global climate change can be observed by extreme weather and temperature fluctuations. Consequences of climate change include, but are not limited to, wildfires, catastrophic droughts, and worsening air and water quality (NASA, 2022a). In addition, these unprecedented changes can result in both the loss of habitats and a decrease in the availability of natural resources. This would have a profoundly negative impact on living organisms.

The buildup of greenhouse gases traps heat from the sun in the atmosphere, warming up the planet (NASA, 2022b). This process is known as the greenhouse effect (Figure 1). It has been reported that the ten hottest years on record have been within the last decade (Climate Central, 2022). Carbon dioxide makes up 0.04% of Earth's atmosphere (Cho, 2019). Since 1960, atmospheric carbon dioxide concentration has increased by nearly 0.01%. Although the number seems minuscule, this is about a 33% increase in the amount of carbon dioxide in the atmosphere between 1960 and 2020, meaning a 2% increase each decade. The all-time highest record of emitting CO2 in the atmosphere was in 2019 which was about 43.1 billion tons of CO2 from human activities.

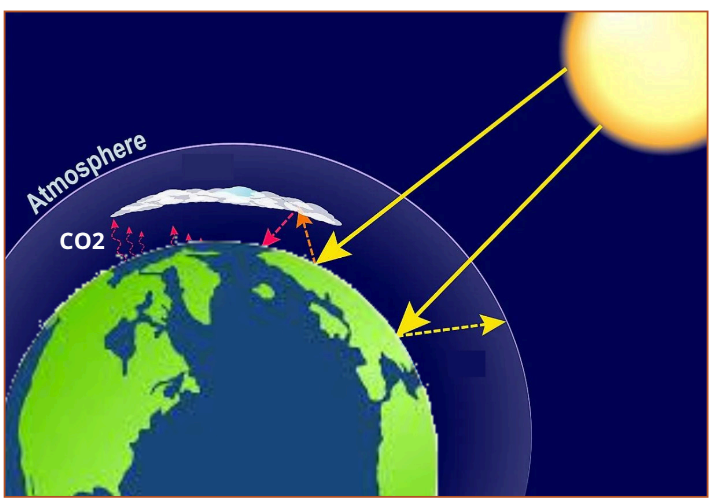

Figure 1. *This figure outlines the greenhouse effect. The sun's rays hit the Earth and heat its' surface. However, a buildup of greenhouse gasses such as carbon dioxide and methane in the atmosphere prevents the light from leaving. This is what causes global warming.*

As a potential solution to combat the effects of CO2 emissions into the atmosphere along with the possible hazardous effects of chemical air fresheners, the AliveFRESH project proposes a biological air freshener. This air freshener will not only provide a pleasant rose-like aroma but will also simultaneously act as a way to decrease the amount of carbon dioxide in the air. *Chlamydomonas reinhardtii* was chosen as the chassis for this project. *C. reinhardtii* is a single-cell green alga that is highly adaptable to environmental changes and is photoautotrophic, meaning it can produce its own using inorganic matter (Merchant *et al.*, 2007). The reason for choosing this chassis is its pre-existing, natural methylerythritol phosphate (MEP) pathway and its ability to convert carbon dioxide into glucose, as well as water into oxygen via photosynthesis.

Photosynthesis is the process of converting water and carbon dioxide into oxygen and glucose using sunlight. The first reaction of photosynthesis is the light-dependent reaction (Figure 2), where light energy converts into chemical energy by producing adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). Both ATP and NADPH molecules can store energy and are transferred to be used in the Calvin cycle. During the Calvin cycle, the second reaction of photosynthesis occurs where carbon dioxide is consumed, and glyceraldehyde-3-phosphate (G3P) is produced. G3P is a versatile sugar that is the starting point for many biosynthetic pathways (including the production of glucose).

Commercially available air fresheners contain toxins such as benzene, formaldehyde, toluene, m,p-xylene, and phthalates. These substances are known as volatile organic compounds (VOCs) and pollute the indoor air of homes. The carcinogen formaldehyde was detected most frequently in previous studies, and it was found with greater frequency when the air freshener was used along with other cleaning products in everyday household use (SCHER, 2006). The United States Food and Drug Administration does not require air freshener manufacturers to disclose some of the chemicals used, many of which have been linked to devastating health impacts. These chemicals include benzene (reproductive toxin), parabens (linked to breast cancer and hormone disruption), toluene (developmental toxin), and xylene (linked to central nervous system depression). Even air fresheners that are labeled as "all-natural" or "clean" include such toxins. One study found that 86% of these "all-natural" air fresheners included phthalates, which are linked to breast cancer and other impacts leading to endocrine disruption (Cohen *et al.*, 2007). These hazards would be prevented with *C. reinhardtii* as a truly natural alternative.

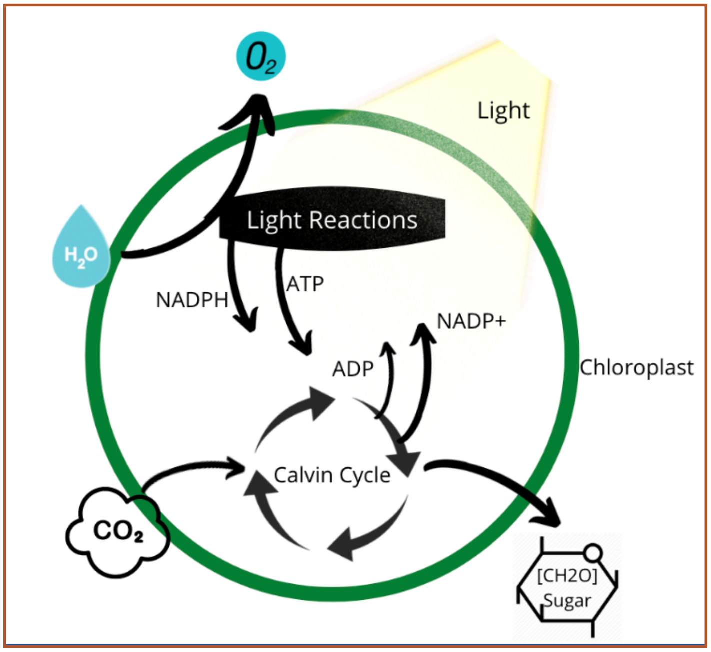

Figure 2. *The Calvin cycle is the second reaction of photosynthesis. Carbon dioxide is consumed, and glyceraldehyde-3-phosphate (G3P) is produced. Water is also split to produce oxygen as a byproduct.*

Glucose can be an energy source for many organisms, including *C. reinhardtii.* This is because it serves as the starting molecule for glycolysis, which in the Chlamydomonadaceae family, takes place in the cytosol (Mitchell *et al.*, 2005). This process takes the glucose created from photosynthesis and produces pyruvate. This pyruvate, as well as G3P created in the Calvin cycle, is a precursor to the MEP pathway. Using these two compounds, the MEP pathway can work and produce isopentenyl pyrophosphate (IPP). Furthermore, IPP and DMAPP are used together as precursors in the presence of prenyltransferases to be converted into intermediates such as geranyl diphosphate (GPP) (Shah *et al.*, 2019), which facilitates the targeted production of the monoterpene geraniol.

Systems Level

AliveFRESH is a simple yet innovative solution to mitigate the current climate change crisis and serves as a natural replacement for harmful commercial air fresheners. This system would take advantage of the existing biological pathways within the selected chassis - *C. reinhardtii.* The engineered organism is autotrophic, and the photosynthetic processes it displays would prove useful. The Calvin cycle naturally converts carbon dioxide into sugar. The sugar product will then be diverted to the MEP pathway to produce IPP, the precursor to GPP production. The team intends to re-engineer *C. reinhardtii* to manufacture geraniol from GPP. This would produce a pleasant rose-like scent (Figure 3). The team will keep the transformed chassis in a container with growth media and a removable lid. Minimal care would be required for a consumer who purchases the product. AliveFRESH only needs to be placed in direct sunlight and requires the replenishment of nutrients and water routinely.

Device Level

This system proposes synthesizing the monoterpene geraniol in *C. reinhardtii.* The process would be made possible by the native MEP pathway found in the organism. The biosynthesis of geraniol would begin

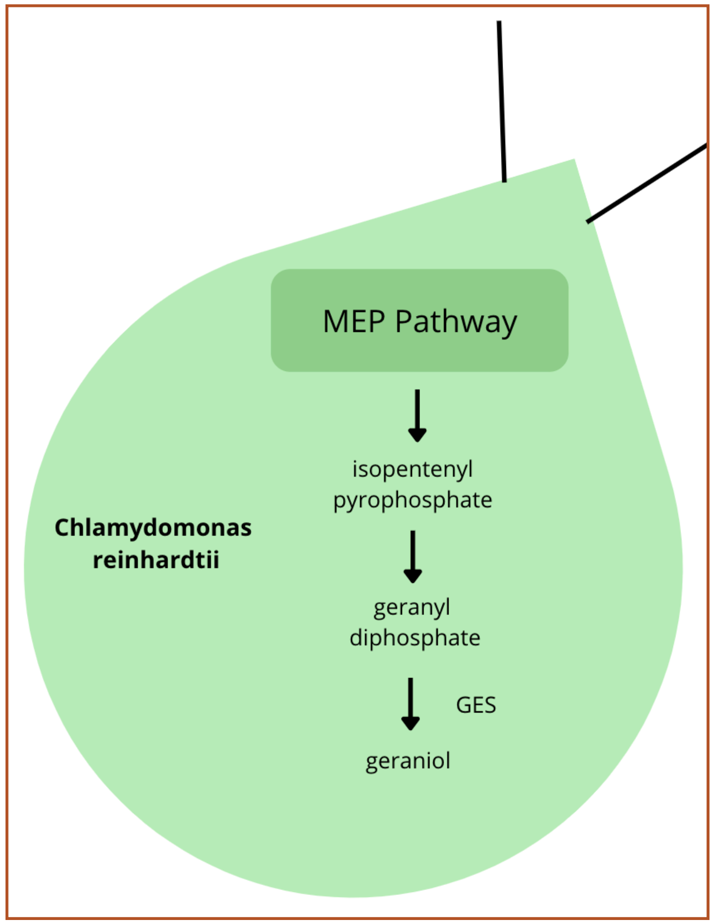

Figure 3. *This is a Systems Level Description of AliveFRESH. Isopentenyl pyrophosphate, the product of the native MEP pathway, would be converted to geranyl diphosphate, the precursor to geraniol. With geraniol synthase, this would convert into geraniol and produce a pleasant scent.*

with the Calvin cycle, which is feasible due to the photosynthetic nature of *C. reinhardtii.* The pyruvate formed from this cycle would feed into the MEP pathway. The following enzymes are required to synthesize IPP: ispC, ispD, ispE, ispF, ispG, and ispH. At this point, GPP synthase would facilitate the reaction between IPP and dimethylallyl diphosphate (DMAPP) to produce GPP, the precursor to geraniol (BioCyc, 2022). The addition of geraniol synthase would complete the process, resulting in a monoterpene with a pleasant scent, which would resemble roses (Figure 4).

Parts Level

The production of geraniol by the MEP pathway requires adding a non-native genetic device. This device would be constructed from a stationary phase promoter, a ribosomal binding site, a GES translational unit, a red fluorescent protein gene with an attached ribosomal binding site, and finished by a double terminator (Figure 5). A stationary phase promoter was chosen because the geraniol production will reach its peak, while the number of living cells plateaus. The ribosomal binding site would be used to control the rate of the geraniol synthase gene translation. The purpose of the red fluorescent protein (BBa_K093005) is to act as a genetic marker to assist in determining the successful transformation of the *C. reinhardtii* cells. Cells that have successfully taken up the genetic construct will fluoresce red when placed under ultraviolet light. The team designed a plasmid (Figure 6) using the backbone pBS-67Sma obtained from the Addgene website as a suitable candidate for transgene integration. It also confers ampicillin resistance, which fits the needs of this current project. This proposed plasmid would be assembled using Gibson assembly and transformed into *C. reinhardtii* for monoterpene production.

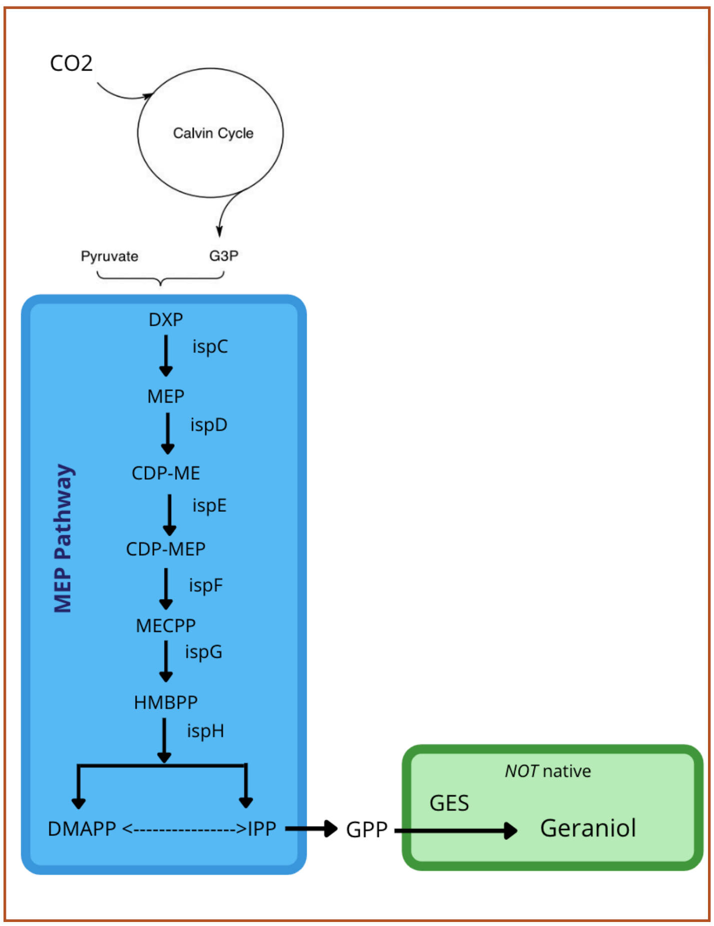

Figure 4. *This is a general description of the AliveFRESH system. First, CO_2 would be converted to G3P by the Calvin cycle. It would then be transformed by the native MEP pathway (colored in blue) to IPP. This would then be converted to GPP. Finally, with the addition of the non-native GES (colored green), GPP would be converted to geraniol. This monoterpene has a floral scent.*

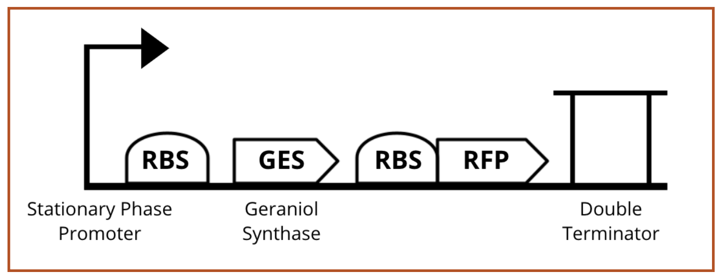

Figure 5. Parts Level Description of AliveFRESH. *This device would be constructed from a stationary phase promoter, a ribosomal binding site (BBa_J01010), a GES translational unit (BBa_K727006), a red fluorescent protein gene with an attached ribosomal binding site (BBa_K093005), and finished by a double terminator (BBa_B0015).*

Safety

For the AliveFRESH experiment, precautions will need to be taken to ensure the safety of all members. The appropriate lab equipment and safety gear will be worn during all experimentation. According to the MSDS safety sheet, geraniol has low mammalian toxicity (TerpeneTech, 2020), but it can be dangerous if it comes into contact with skin (ThermoFisher Scientific, 2011). Therefore, any experiments involving this chemical will be performed under a fume hood with ventilation. Non-latex gloves and aprons will be used to ensure that none of the cultures or samples will come into contact with any part of skin. Under normal conditions, respiratory protection is unnecessary. However, goggles will be worn during experiments to avoid any eye irritation.

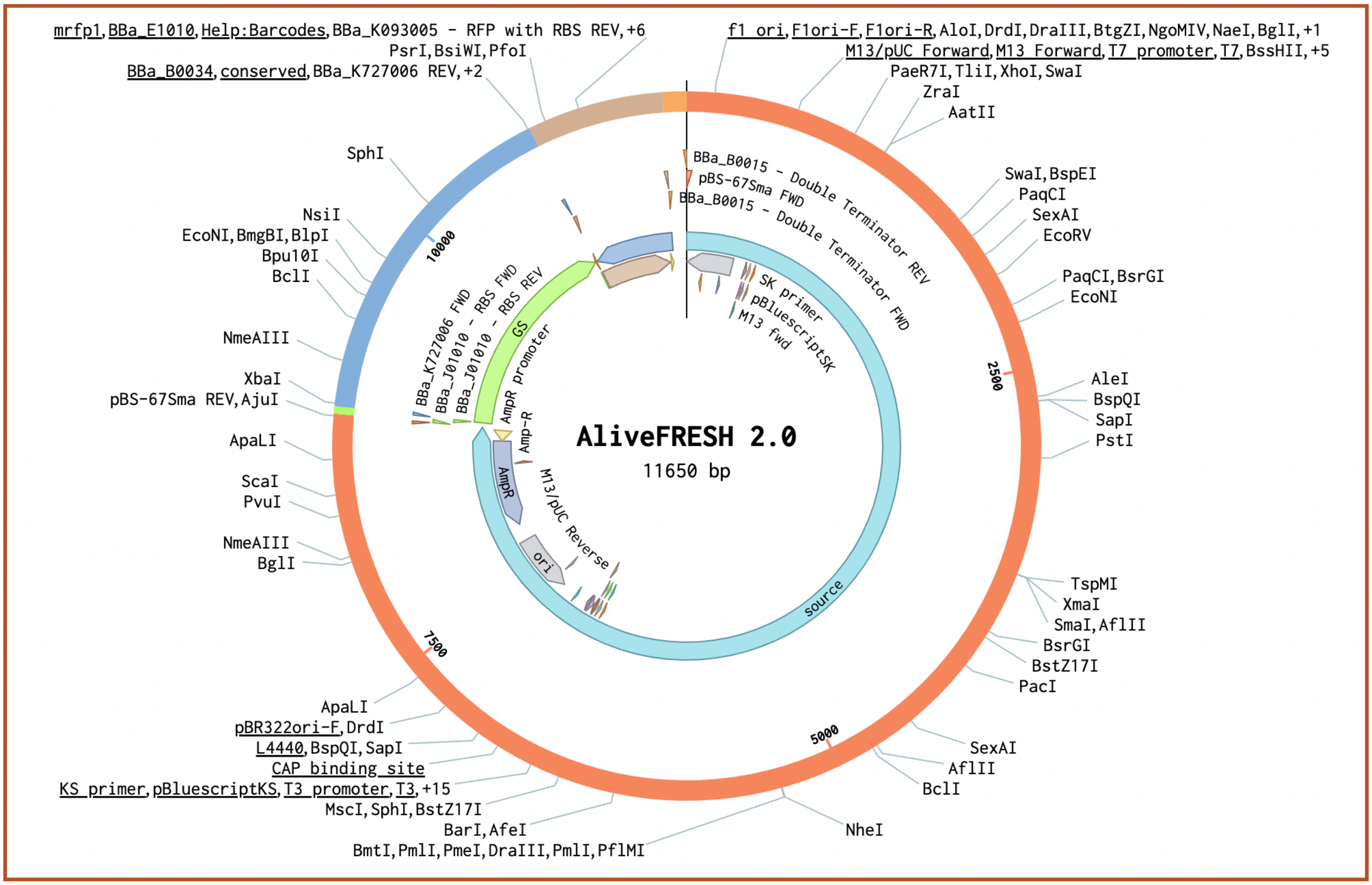

Figure 6. *AliveFRESH plasmid. It uses the backbone pBS-67Sma imported from Addgene. This backbone was chosen because it was designed with the intent of transforming Chlamydomonas for transgene integration. It also possesses ampicillin resistance. In addition, it contains a genetic device designed by the team to produce geraniol (Figure 5).*

Discussions

Carbon dioxide is a harmful greenhouse gas that has damaged ecosystems and increased temperatures across the planet. The team will use *C. reinhardtii* as the chassis in this biodesign because it is photosynthetic and removes carbon dioxide from the atmosphere. *C. reinhardtii* expresses a native MEP pathway that is initiated by the products of the Calvin cycle and glycolysis (G3P and pyruvate, respectively). The aim of this project is to build a device that produces the floral scented geraniol while utilizing carbon dioxide. Because of this, AliveFRESH serves as an environmentally-friendly alternative to current air fresheners that can harm the environment. An additional advantage of AliveFRESH is that this kind of air freshener would not have to be purchased like other products currently on the market that create unwanted waste. Using *C. reinhardtii* provides an alternative to the artificial and toxic air fresheners that currently exist. In order to sustain the air freshener culture, it must be kept in optimal growth conditions, including a temperature between 20-32 degrees Celsius and within a 5.5 to 8.5 pH range (Messerli *et al.*, 2005 and Xie *et al.*, 2013). As with all living organisms, *C. reinhardtii* must be fed. The culture will be grown in Hutner's Media (Hutner *et al.*, 1950). This system could then be used with ease by the general public.

Next Steps

We will transform *C. reinhardtii* through electroporation for geraniol transformation. According to a study by Brown *et al.* (1991), DNA can be integrated using plasmids in the process known as electroporation which opens small holes in the cell membrane to allow DNA to be inserted directly into the genome. *Chlamydomonas* is known to have plasmids and can be transformed using electroporation. The team understands the obstacles associated with producing terpenes in non-terrestrial plant hosts. The strategies proposed here provide an entry point for future work because photosynthetic microbe model organisms would be beneficial to this industry.

The monoterpene, geraniol, would be detected through a scent test. An olfactometer would be the most useful tool for determining the intensity of the scent. Additionally, detection of successful *Chlamydomonas* transformants will be screened for the presence of red fluorescent protein (RFP). RFP can be produced by algae including *C. reinhardtii* (Molino *et al.*, 2018). According to Liu *et al.* (2013), the MEP pathway produces an adequate amount of GPP to supply the non-native genetic device that converts GPP to geraniol in the presence of GES. If there was insufficient GPP production, other native pathways that produce G3P and pyruvate to supply the MEP pathway would be considered. For example, combining the products of the Entner-Doudoroff (EDP) and pentose phosphate (PPP) pathways would optimize IPP production, and since IPP is converted to GPP, there would be more GPP to produce geraniol.

The optimum temperature range for raising *C. reinhardtii* is between 15 to 37℃. At this temperature, their life span ranges from 14 to 34 hours (Vítová *et al.*, 2011). *C. reinhardtii* is very easy to culture and maintain because of its short life span, quick growth, and small size (Rochaix, 2013). Because the life span is short, AliveFRESH would need to be frequently replenished to allow for continued growth .

Author Contributions

B.C., G.C., Y.C., E.F., A.J., N.M., and M.P. conducted background research. Y.C., G.C., E.F., and M.P. wrote and edited the abstract. B.C., G.C., Y.C., E.F., A.J., N.M., and M.P. wrote the manuscript, which was edited by B.C., G.C., Y.C., E.F., N.M., M.P., and Y.C. created and developed the video and A.J. created the manuscript graphics. Y.C. designed the plasmid and E.F. compiled resources.

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