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Ethylene-Producing Bacteria That Ripen Fruit

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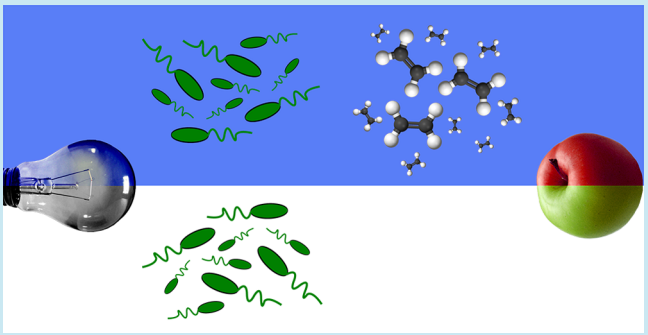
ABSTRACT: Ethylene is a plant hormone widely used to ripen fruit. However, the synthesis, handling, and storage of ethylene are environmentally harmful and dangerous. We engineered *E. coli* to produce ethylene through the activity of the ethylene-forming enzyme (EFE) from *Pseudomonas syringae*. EFE converts a citric acid cycle intermediate, 2-oxoglutarate, to ethylene in a single step. The production of ethylene was placed under the control of arabinose and blue light responsive regulatory systems. The resulting bacteria were capable of accelerating the ripening of tomatoes, kiwifruit, and apples.



KEYWORDS: *ethylene, fruit ripening, bioengineering, YF1, blue photoreceptor, iGEM*

Ethylene is a plant hormone that triggers fruit ripening, resulting in the degradation of starch to simpler sugars, a softening of the fruit, and a change in skin color.[1] Ripening can be induced by endogenous and exogenous ethylene,[2] which is why many commercially purchased fruit are ripened post-harvesting by exposure to ethylene. While effective, there are at least two limitations with the current ethylene-based methods. First, ethylene is typically produced through the steam cracking of fossil fuels, an environmentally harmful process. Second, ethylene is explosive, making the use of pressurized canisters of ethylene to control fruit ripening unsafe.

We sought to develop an environmentally friendly and safe way of controlling fruit ripening by engineering *E. coli* to synthesize and release ethylene. The three-step plant ethylene synthesis pathway was not exploited,[2] because the plant pathway produces the toxic byproduct hydrogen cyanide in addition to ethylene. Instead, a single enzyme from *Pseudomonas syringae* pv phaseolicola was inserted in *E. coli*. This enzyme, ethylene forming enzyme (EFE) or 2-oxoglutarate oxygenase/decarboxylase, catalyzes the conversion of a molecule of the citric acid cycle, 2-oxoglutarate, to gaseous ethylene, which subsequently permeates across the bacterial membrane.[3−5] EFE expression was controlled by arabinose and blue light.

RESULTS AND DISCUSSION

Pseudomonas syringae pv phaseolicola PK2 *EFE* was codon optimized for *E. coli* and synthesized by GenScript with an upstream ribosome binding site (RBS). The sequence was subcloned into pSB1C3 via standard BioBrick assembly (BBa_K1065000). Subsequently, an arabinose responsive promoter (BBa_K731201) and double transcriptional terminator (BBa_B0015) were placed upstream and downstream, respectively, of the RBS-EFE construct. *E. coli* NEB10*β* were transformed with this arabinose inducible device (BBa_K1065001) and grown in LB at 37 °C to an optical density (OD) at 600 nm of 0.5. Four h after the addition of 5 mM arabinose, ethylene production was detected with an Agilent 3000A Micro Gas Chromatograph. After constructing a standard curve, the detected ethylene was found to correspond to 101 ± 15 ppm (Figure 1a). The uninduced sample did not produce detectable levels of ethylene. To better understand which conditions improved ethylene production, a kinetic analysis of *E. coli* induced at two different cell densities was performed. *E. coli* were grown to an optical density (OD) of 0.5 and 0.8, induced with 5 mM arabinose, and ethylene production monitored by gas chromatography. The higher cell density sample produced approximately 3-fold more ethylene than the lower cell density sample (Figure 1b). Both samples reached maximum ethylene output within 2.5 h after induction.

Next, ethylene synthesis systems regulated by light instead of arabinose were built. YF1[6] (BBa_K592004) was used as the light sensor. In the presence of blue light, the kinase activity of YF1 is reduced resulting in a decrease of phosphorylation of the response regulator FixJ (BBa_K592005). Phosphorylated FixJ activates expression from the fixK2 promoter (BBa_K592006).

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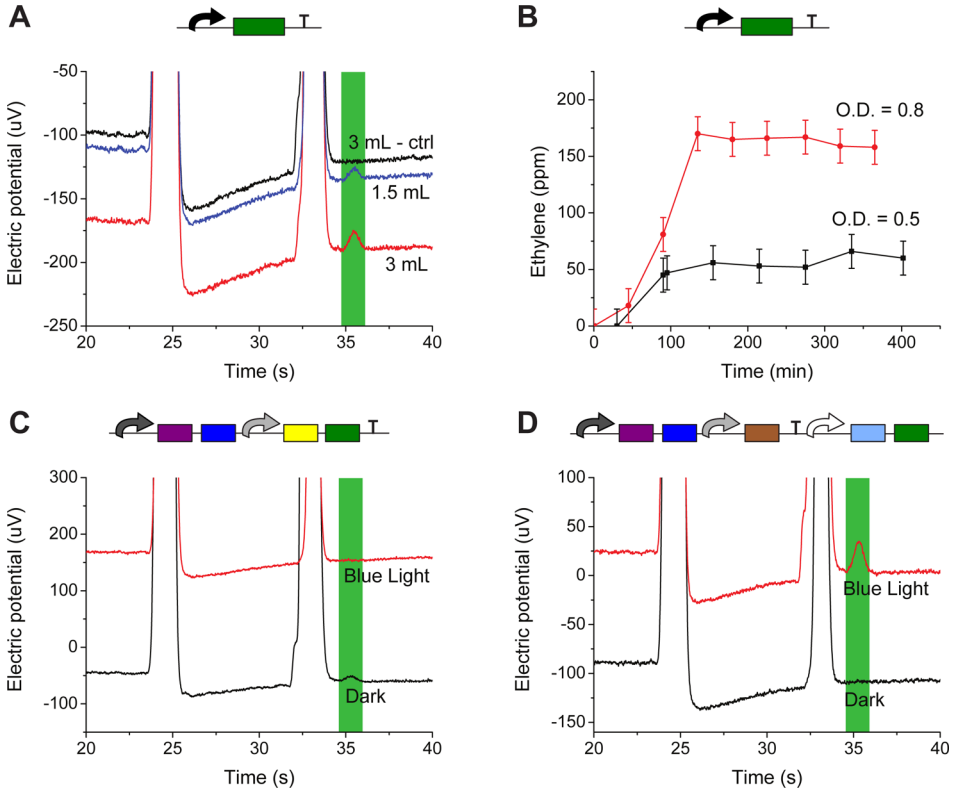

Figure 1. Bacterial production of ethylene. (A) Gas chromatogram of ethylene produced from *E. coli* expressing EFE under arabinose control. *E. coli* NEB10*β* BBa_K1065001 were induced with 5 mM arabinose at an OD of 0.5 and analyzed 4 h after induction by gas chromatography. The ethylene produced from 1.5 mL (blue) and 3.0 mL (red) cultures was quantified. An uninduced 3 mL culture was used as a control (black). (B) Kinetic profile of ethylene production from *E. coli* expressing recombinant EFE under arabinose control. *E. coli* NEB10*β* BBa_K1065001 (3 mL) was grown to either an OD of 0.5 (black) or 0.8 (red) prior to induction with 5 mM arabinose and monitored by gas chromatography. (C) Gas chromatogram of ethylene produced from *E. coli* with blue light inhibited EFE expression. *E. coli* NEB10*β* BBa_K1065309 were cultured under blue light until an OD of 0.7. Subsequently, 3 mL aliquots were either incubated in the dark (black) or under blue light (red) overnight. (D) Gas chromatogram of ethylene produced from *E. coli* with blue light activated EFE expression. *E. coli* NEB10*β* BBa_K1065311 was cultured as in part C. All cultures were grown in LB supplemented with 34 *μ*g/mL chloramphenicol at 37 °C. Ethylene elutes from the HP-PLOT U column (Agilent) after 35 s (highlighted in green). The retention time of ethylene was confirmed with a control sample of pure ethylene. A schematic above each panel represents the exploited construct with each color representing a different part: black, BBa_K731201 (araC-pBAD); green, BBa_K1065000 (EFE); dark gray, BBa_R0010 (lacI promoter); purple, BBa_K592004 (YF1); blue, BBa_K592005 (*fixJ*); gray, BBa_K592006 (fixK2 promoter); yellow, BBa_K592010 (amilGFP); brown, BBa_C0051 (cI); white, BBa_R0051 (lambda cI regulated promoter); light blue, BBa_K592009 (amilCP). The T represents BBa_B0015 (terminator).

Genes coding for the yellow chromoprotein amilGFP (BBa_K592010) and EFE were placed downstream of the FixJ responsive fixK2 promoter. In this arrangement, ethylene should be produced in the dark and not in the presence of blue light. *E. coli* NEB10*β* were transformed with the final construct (BBa_K1065309) and grown in LB at 37 °C to an OD of 0.7, after which an aliquot (3 mL) was placed in the dark. Ethylene (37 ± 15 ppm) was produced in the dark (Figure 1c). No detectable ethylene was found from cultures kept in the light.

To build a device that activates ethylene production upon exposure to light, an inverter composed of the cI repressor (BBa_C0051) and a lambda cI regulated promoter (BBa_R0051) was incorporated into the genetic construct. Here, the blue chromoprotein amilCP (BBa_K592009) and EFE were under the control of the lambda cI regulated promoter. *E. coli* NEB10*β* transformed with this construct (BBa_K1065311) containing the inverter were grown in the dark in LB at 37 °C to an OD of 0.7 and then exposed to a 1 W blue LED overnight. Ethylene (92 ± 15 ppm) was produced with blue light exposure, whereas no ethylene was detected with a control reaction kept in the dark (Figure 1d). It should be noted that although both inverter-lacking and inverter-containing systems worked, difficulties in reproducibility were encountered. This is in contrast to the performance of the arabinose controlled ethylene synthesis device, which always gave data consistent with the engineered activity.

Having demonstrated that *E. coli* could be engineered to synthesize and release ethylene in a controlled manner, we next sought to determine if our engineered bacteria could accelerate fruit ripening. *E. coli* carrying the arabinose inducible device BBa_K1065001 were incubated in an Erlenmeyer flask connected to an airtight jar containing the fruit for 24 h (Figure 2a). For each experiment, two control reactions were run. One control lacked a bacterial culture and the other exploited uninduced bacteria. After incubation with induced bacteria, the skin and pulp color change of cherry tomatoes was consistent with accelerated ripening (Figure 2b). Ethylene producing bacteria were also used to ripen heirloom tomatoes, kiwifruit, plums, bananas, and apples. Spectrophotometric measurements confirmed that the pulp of kiwifruit was of a darker green than control samples (Figure 2c).

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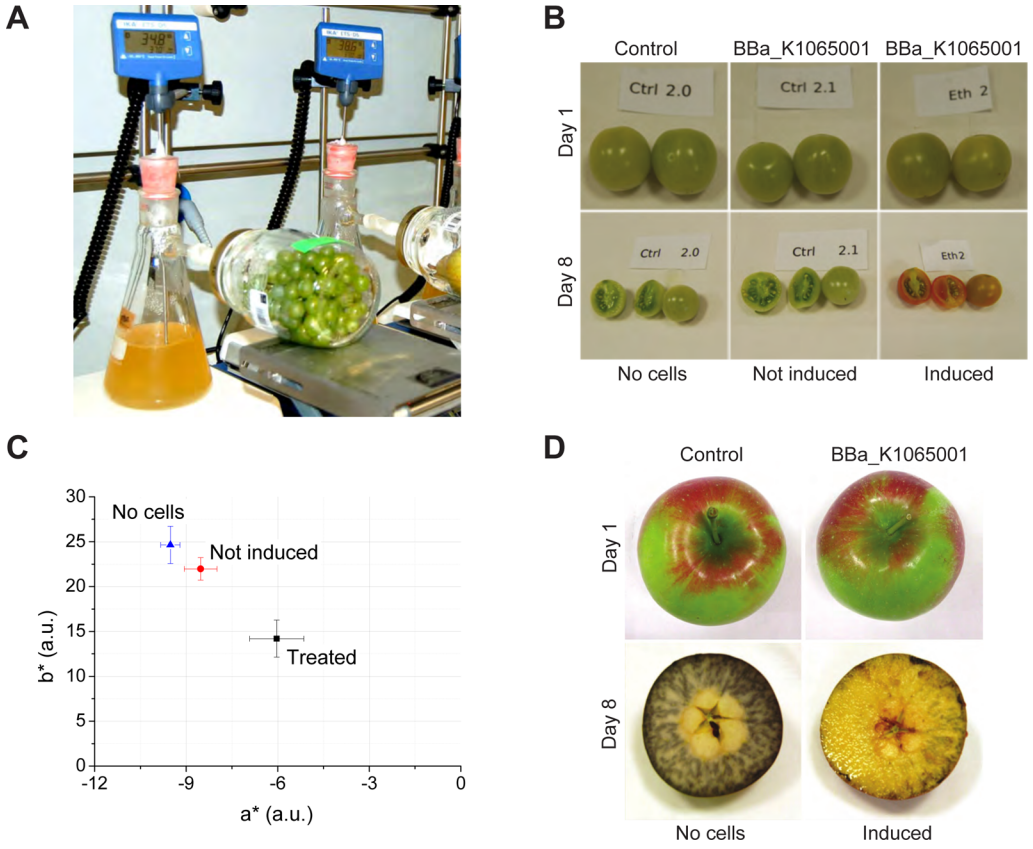

Figure 2. *E. coli* produced ethylene ripens fruit. (A) The experimental setup for bacterially induced fruit ripening. (B) Cherry tomato ripening was accelerated with *E. coli* NEB10β BBa_K1065001. The cherry tomatoes were exposed to the bacterially produced ethylene in 0.5 L glass containers for 24 h. Eight days later the fruit was removed from the container and sliced. (C) Kiwifruit color development is accelerated with *E. coli* NEB10β BBa_K1065001. Fruit color was measured with a HR2000+ spectrometer equipped with a fiber optic reflection probe (Ocean optics). Data are expressed in red (a*) and yellow (b*) absorbance. Smaller values of a* and b* signify more green and less yellow, respectively. Kiwifruit treated for 8 days with bacterially produced ethylene (black) were darker green than incubation with uninduced bacteria (red). A control kiwifruit sample was not incubated with bacteria (blue). (D) A starch-iodine test demonstrated ripening of apples. Apples were exposed to bacterially produced ethylene for 3 days and left in the glass containers for an additional 5 days. Apples not incubated with bacterially produced ethylene were used as a control. Apples under both conditions were sliced in half and then submerged in Lugol's solution (iodine–potassium iodide, Sigma-Aldrich) for 1 min. The production of blue-black color indicates the presence of starch. The experiment was repeated twice yielding the same qualitative results.

In addition to color changes, ripening results in starch degradation and softening of the fruit. To determine if ethylene producing *E. coli* induce both of these changes, starch degradation was evaluated by an iodine assay and softness probed by compression strength tests. Iodine in potassium iodide reacts with starch to give a purple-black color. The iodine assay showed much less color development for apples exposed to ethylene producing bacteria than control samples (Figure 2d). Similarly, kiwifruit treated with ethylene producing bacteria were 14- to 25-fold less firm than control samples. The breaking point, measured with an Instron 4502, for kiwifruit exposed to induced bacteria, uninduced bacteria, and kiwifruit not exposed to bacteria were $3.1 \times 10^{-5} \pm 2.4 \times 10^{-6}$ kN/mm^2, $4.5 \times 10^{-4} \pm 1.8 \times 10^{-5}$ kN/mm^2, and $7.6 \times 10^{-4} \pm 7.3 \times 10^{-5}$ kN/mm^2, respectively. The difference between the two control reactions was likely due to CO_2 produced by the bacterial culture, which is known to affect fruit ripening.[7]

METHODS

Plasmid Assembly. All parts were from the registry of standard biological parts except for *P. syringae* pv phaseolicola PK2 EFE, which was synthesized by GenScript. All constructs were assembled via standard BioBrick assembly. DNA inserts and plasmid backbones were digested overnight at 37 °C. pSB1C3 was linearized by PCR with Phusion polymerase (New England BioLabs) using primers containing BBa_G00000 prefix and BBa_G00001 suffix sequences and purified with the Promega Wizard SV Gel and PCR Clean-Up System following the manufacturer's instructions. Linearized pSB1C3 was digested overnight and subsequently treated with 20 units DpnI. After ligation with T4 DNA ligase at room temperature for 1 h, $CaCl_2$ competent *E. coli* NEB10β were transformed with ligated DNA samples and plated on LB supplemented with 34 μg/mL chloramphenicol. All constructs were verified by sequencing at Genechron (Ylichron, ENEA).

Ethylene Production. An overnight culture of *E. coli* NEB10β was diluted 100-fold in LB supplemented with 34 μg/mL chloramphenicol and grown to an OD at 600 nm of 0.5–0.8. A 3 mL aliquot was then induced with 5 mM arabinose and placed in a 15 mL vial sealed with a rubber septum. The sample was continuously stirred and connected to a Micro Gas Chromatograph Agilent 3000A equipped with a HP-PLOT U column (Agilent). Measurements were taken every 45 min. For blue light responsive constructs, cultures were incubated in the dark until OD 0.7 and then split in two. Half of the culture remained in the dark and the other half of the culture was

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exposed to a blue (470 nm) LED (Osram Oslon) overnight. Secreted ethylene was quantified by gas chromatography as described above.

Fruit Ripening. A 300 mL culture of *E. coli* NEB10β was prepared as described above and placed in a 0.5 L Erlenmeyer flask containing a stir bar and connected via rubber tubing to a desiccator chamber containing the fruit. The culture was kept at 37 °C. Each day the bacterial culture was discarded and replaced with fresh cells. The total reaction time was 6−8 days, as indicated. Fruit were then cut and analyzed by several methods. Starch degradation was evaluated by immersing samples in Lugol's solution (Sigma-Aldrich) for 1 min and then immediately dried with a paper towel. Coloration was qualitatively evaluated by eye. Softening of fruit was investigated with a 4502 Instron tensile strength test system. Fruit were cut into 12 mm × 12 mm × 12 mm cubes. The plate approach speed was 1.3 mm/min. Quantitative evaluation of coloration exploited a HR2000+ miniature spectrometer (Ocean Optics Inc., Dunedin, FL) equipped with a fiber optic reflection probe (Ocean Optics Inc., Dunedin, FL). Data were analyzed with Spectra Suite software (Ocean Optics, Dunedin, FL).

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Author Contributions

This project was carried out by the UNITN-Trento 2013 iGEM team. B.A., F.D., G.G., C.M., M.P., T.P., E.T., V.V. equally contributed to the molecular and microbiological experiments. T.P. ran the gas chromatography experiments with the support of D.A. and F.D. Fruit ripening was by B.A. and T.P., and the quantification of ripening was by B.A., T.P., A.D., and G.F. F.D., G.G., C.D., O.J., and S.S.M. wrote the manuscript. C.D. participated in the daily experimental planning. C.D., P.T., S.S.M., and O.J. helped coordinate the study.

Notes

The authors declare no competing financial interest.

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