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University of Nevada, Reno

# Biofortification of rice though CBM21 fusion proteins: A demonstration with a starch binding red fluorescent fusion protein

A thesis submitted in partial fulfillment of the

Requirements for the degree of

Bachelor of Biochemistry and Molecular Biology and the Honors Program

by

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We recommend that the thesis
prepared under our supervision by

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be accepted in partial fulfillment of the

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BACHELOR OF SCIENCE

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Christie Howard, Ph.D., Thesis Advisor

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**Abstract**

Rice is a major form of sustenance for over half of the world's population. White rice is a poor source of nutrients. Even though rice can be fortified using vitamin powders and other such methods, these methods have had limited success because many vitamins are leeched away during the washing process prior to cooking. The overall goal of the project is to create a recombinant protein containing a starch binding domain fused with one of various nutrient binding domains, such as vitamin B12 or thiamine. This fusion protein can attach nutrients to rice grains and provide a way to fortify rice without losing the supplement during washing. A protein consisting of a starch binding domain(cbm21) and a red fluorescent protein was used as a proof of concept for the effectiveness of the starch-binding capabilities of the fusion proteins. The results of this experiment show that the starch binding domain sticks to rice. Interestingly, red fluorescent protein sticks to rice without the need for a starch binding domain, though rather less effectively.

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## Introduction

Over half of the world's population depends on rice as a major source of calories. The majority of the rice eaten is polished white rice (Bhullar and Gruissem 2012). Though brown rice is healthier than white rice, most of the world prefers white rice because of personal and cultural preferences and its ability to be stored for long periods of time (Ellis *et. al*. 1986). White rice is created from raw rice through the removal of the hull, bran, and germ layers. This process results in a large loss of nutritional value as the bran layer contains the majority of rice's nutrition. The leftover product is mostly starch.

Proximate composition and vitamin/mineral content of brown and white rice.

| Item | Brown rice | White rice |
|---|---|---|
| Crude protein, g/100g | 7.1-8.3 | 6.3-7.1 |
| Crude fat, g/100g | 1.6-2.8 | 0.3-0.5 |
| Crude fiber, g/100g | 0.6-1.0 | 0.2-0.5 |
| Available carbohydrates, g/100g | 73-87 | 77-89 |
| Neutral detergent fiber, g/100g | 2.9-3.9 | 0.7-2.3 |
| Thiamine, mg/100g | 0.29-0.61 | 0.02-0.11 |
| Riboflavin, mg/100g | 0.04-0.14 | 0.02-0.06 |
| Niacin, mg/100g | 3.5-5.3 | 1.3-2.4 |
| α-tocopherol, mg/100g | 0.90-2.50 | 0.075-0.30 |
| Calcium, mg/100g | 10-50 | 10-30 |
| Phosphorus, g/100g | 0.17-0.43 | 0.08-0.15 |
| Phytin P, g/100g | 0.13-0.27 | 0.02-0.07 |
| Iron, mg/100g | 0.2-5.2 | 0.2-2.8 |
| Zinc, mg/100g | 0.6-2.8 | 0.6-2.3 |

SOURCE: FAO, 1994 cited from Juliano, 1985; Eggum, Juliano, and Maniningac, 1982; Pedersen and Eggum, 1983.

**Table 1: Nutritional content of brown and white rice:** This graph shows the approximate composition and vitamin and mineral content of both brown and white rice. White rice has less nutrition than brown rice due to the loss of the bran layer during the milling process (Pedersen, B. & Eggum 1983).

In many countries, nutrient deficiencies in staple foods have been minimized through fortification (Alvali *et al.* 2012). For example, wheat is fortified in the United States. However, rice fortification using standard techniques, for example dusting, has had limited success around the world as many cultures wash their rice before cooking (Alvali *et al*. 2012). This washing removes the dirt as well as the added nutrients. Genetic engineering is one method that has been chosen to add to rice's nutritional value. An example of this is golden rice. Golden rice has been

engineered to produce beta-carotene to help with vitamin A deficiency (Mayer, J.E. 2007). Sadly, golden rice is not being utilized widely because of public distrust of genetically modified foods and the countries that produced them (Tenbult, P., *et al*. 2008). Some of the distaste with genetically modified food is due to concerns over the golden color of the rice, worries of horizontal gene transfer, and sensationalism.

The creation of a rice and nutrient binding protein that remains bound through washing circumvents the washing issue. This solution can even avoid the problems with genetically engineering rice by not engineering the rice itself. Instead, *Escherichia coli* can be engineered to create recombinant protein and the protein can be purified and added to rice after the polishing process.

*Rhizopus oryzae* is an organism that produces glucoamylase enzyme. This enzyme aids in starch breakdown by binding to starch and providing optimal interaction with enzymes that break down the starch (Chou, W.I. *et al* 2006). The starch binding domain of the protein is encoded for by the gene cbm21 and binds strongly enough to be used as a fusion purification tag with amylose columns (Lin, S., *et al* 2009). Therefore, this starch binding domain could perceivably be used in a fusion protein to bind nutrients to rice.

In order to demonstrate that the starch binding domain can bind to polished white rice and determine how effectively it can bind, a fusion protein consisting of the starch-binding domain encoded for by cbm21 with a red fluorescent domain encoded by the mRFP1 gene was produced and tested. This fusion protein construct binds to starch and fluoresce when exposed to UV light. The red fluorescent protein allowed for clear visualization of whether or not binding occurs by the cmb21 domain and provided proof of concept for the project.

# Methods

## Fusion of cbm21 with mRFP1 using In-Fusion Cloning

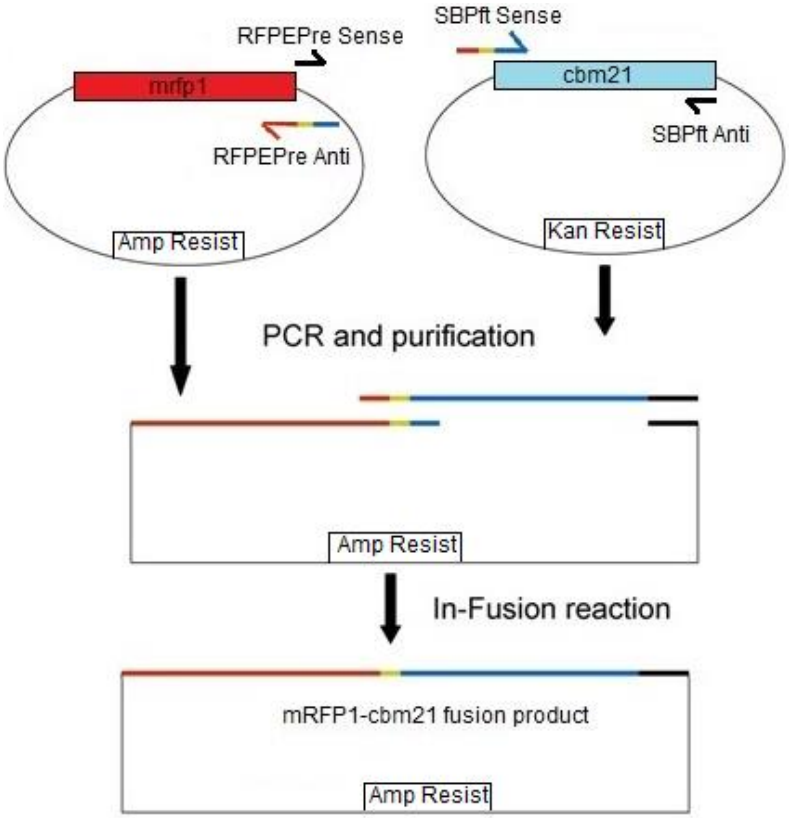


**Figure 1: In-Fusion Cloning Overview**: The plasmid containing mRFP1, part BBa_J23117, was cloned using primers RFPEPre Sense and RFPEPre Anti to create a linear version of the entire plasmid with an added fusion tag after the mrfp1 gene. The plasmid, BBa_931000, was cloned to extract only the gene cbm21 with an added fusion tag before it. The In-Fusion method allows them to be fused using the tags.

Two parts containing the starch binding domain (cmb21) and red fluorescent protein (mRFP) from iGEM were transformed into Top10 competent cells from Invitrogen. Each was plated and cultured in Terrific broth that contained the antibiotic with which they are resistant (Figure 2) at 37$^{o}$C overnight. Both cultures were then processed to isolate their plasmid DNA through a Qiagen Miniprep kit.

| Part Name | Gene of Interest | Antibiotic Resistance |
|---|---|---|
| a. BBa_931000 | cmb21 | Kanamycin resistance |
| b. BBa_J23117 | mRFP1 | Ampicillin resistance |

**Table 2: iGEM Biobricks:** Parts are taken from the iGEM standard parts registry. The gene cmb21 codes for starch binding protein. The gene mRFP1 codes for red fluorescent protein. The plasmids also contain constitutive promoters, ribosome binding sites, and terminators.

To create the red fluorescent protein (RFP) construct and starch binding protein (cmb21 domain, a.k.a. SBP), PCR was used to first isolate both genes and attach fusion tags. The reactions utilized 1 µL each of their appropriate plasmid DNA, either SBP (Figure 2a) or RFP (Figure 2b), 10 µL of Promega GoTaq Green Master Mix, 7 µL of $ddH_2O$, and 1 µL each of the corresponding forward and reverse primers (Table 3 a,b/c,d). A standard PCR reaction of 30 cycles was used with denaturation at 95$^{o}$C for 30 seconds, annealing 55$^{o}$C for 30 seconds, and elongation at 72$^{o}$C for 5 minutes.

| Primer | Sequence | Purpose |
|---|---|---|
| a. RFPEPre Sense60 | TAACGCTGATAG TGCTAGTGTAGATCGCTACTA | Places a fusion tag after mRFP1. Forward direction. |
| b. RFPEPre Anti60 | ACTAGAAGCACCGGT GGAGTGACGACCTTCAGCA | Places a fusion tag after mRFP1. Reverse direction. |
| c. SBPft Sense | TTCGGAGGAAGCCAT ATGGCATCGATTCCGAGCAGC | Places a fusion tag before cbm21. Forward direction. |
| d. SBPft Anti | GAGAAATACTAGATG GGTAGACACTTGGTAGTTCGCGCTA | Places a fusion tag before cbm21. Reverse direction. |
| e. SBP Sense124 | AAGAAAGTGACGGTCGTTTATGC | CBM21 forward primer. |
| f. SBP Anti | CTATCAGCGTTATTAGGTAGACACTTGGTAGTTCGCGCTATTGTTA | Cbm21 reverse primer |

**Table 3: Primer Sequences and Usage:** Each primer was used in PCR to create RFP1 and cbm21 with fusion tags on them. Fusion tags were added after mRFP1 and cbm21.

After PCR purification using a Qiagen Purification Kit, a digestion was set up with 7 µL of the linearized RFP plasmid, 1 µL of DPNI digestion enzyme, 2 µL of Buffer 4 from New England Biolabs, and 0.5 µL of Bovine Serum Albumin. This was allowed to incubate overnight at 37$^{o}$C. The following day the digestion was heat inactivated at 85$^{o}$C for 20 minutes.

The fusion ligation was prepared with 4 µL of the linearized RFP plasmid, 4 µL of the cbm21 insert, 1 µL of ligation enzyme, and 1 µL of buffer from the In-Fusion cloning kit. After twenty minutes of incubation time at room temperature, the fusion product was transformed into Top10 cells, incubated in 250 µL of Super Optimal without Catabolites, or SOC, broth. Then, 100 µL of it was spread on ampicillin plates. The protocol was repeated using a different enzyme for the PCR called LongAmp Taq DNA Polymerase from New England Biolabs.

Eight colonies from the SBP-RFP plate were tested using colony PCR. This was done to confirm that mRFP1 and cbm21 were present in the correct orientation. Two microliters of the colony diluted in dd$H_2O$, 5 µL of GoTaq Green Mastermix, 1 µL each of SBP-sense and SBP-anti primers(Table 3 e,f), and 1 µL of dd$H_2O$ were used in the colony PCR. Once the PCR was completed, the products were placed on agarose gels and electrophoresis was used to detect the presence of our DNA PCR product. Cultures with Terrific broth and ampicillin were prepared for two successful colonies.


**Figure 2: Fusion Product of RFP-SBP:** iGEM part, BBa_J23117, which contains mRFP1 (RFP), with cbm21 (SBP) inserted into it. The expressed fusion protein contains both a red fluorescent domain as well as a starch binding domain.

## Protein Harvest and Usage

One hundred milliliters of RFP-SBP was cultured in ten 10ml tubes. Each culture was then centrifuged and the supernatant discarded. The pellet was resuspended in phosphate buffered saline (PBS). The solutions were then combined, placed on ice, and a sonic lysis machine was used on the solution; 8 cycles for 8 seconds each. The solution was then centrifuged and the supernatant removed. A small amount of rice, ten kernels, was then added to this protein solution and incubated for 5 hours at $10^{o}$C.

## Examination

The rice sample was washed by three methods before examination. The methods consisted of one minute of a swirling method, one minute of a pinching method, and 30 seconds under the tap. The RFP-SBP coated rice was examined under a fluorescent and white light microscope. The red fluorescent protein encoded for by mRFP1 has an excitation wavelength of 584nm and an emission wavelength of 607nm (Nathan C Shaner *et al* 2004).

## Positive Control

Ten tubes of RFP-SBP were cultured and the protein isolated in the same fashion as above. In addition to cultures of RFP-SBP containing cells, cells with RFP alone were cultured and processed identically. The fluorescence of each protein solution was measured and each solution diluted to equalize fluorescence between the two. Rice was treated and examined under a fluorescent microscope (excitation 584 nm, emission 607nm) after both two and four hours of incubation.

## Protein Spray

Water combined with either the RFP or RFP-SBP solutions was applied to three separate dry rice samples with a spraying device. Spraying consisted of three uniform one second sprays to each sample. The sprayed rice was allowed to dry for three hours before being washed with three methods as described above. The three samples were then examined under the fluorescent microscope as done previously.

# Results

## Fusion

The mRFP1-cbm21 construct was grown on an Ampicillin plate in Top10 competent cells. Eight red colonies from the plate were selected and a colony PCR performed to confirm the presence of cbm21, which is 361 base pairs long, using the SBPsense124 and SBPanti primers (Table 3 e,f). The PCR products were run on a 1.2% agarose gel with ethidium bromide in it. Five of the colonies chosen displayed bands between 300 and 400 base pairs in size.

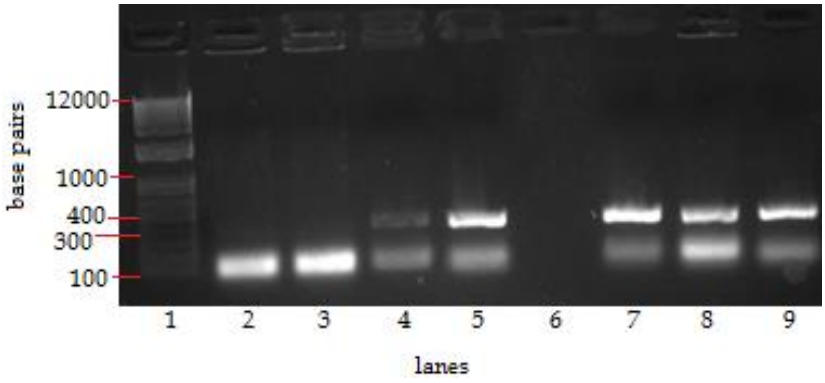


**Figure 3: Agarose Gel Electrophoresis Colony PCR of RFP-SBP genes:** Lane 1: Invitrogen 1KB Plus DNA ladder. Lanes 2: colony 1. Lane 3: colony 2. Lane 4: colony 3. Lane 5: colony 4. Lane 6: colony 5. Lane 7: colony 6. Lane 8: colony 7. Lane 9: colony 8. A PCR of red Top10 competent cell colonies in which RFP-SBP plasmid was transformed was run through electrophoresis on a 1.2% agarose gel with ethidium bromide in it. Colonies in lanes 4, 5, 7, 8, and 9 contain bands of between 300 and 400 base pairs in size.

## Protein Harvest and Usage

When protein was harvested from ten 10ml of the RFP-SBP protein, approximately 10ml of pink solution was the result. This pink protein solution was incubated with washed rice for 5 hours. After this incubation, the rice was washed again before being examined under a fluorescent microscope set to cause RFP to fluoresce. Viewing the protein treated rice next to the untreated rice showed a stark difference in brightness with the RFP-SBP treated rice glowing significantly brighter than the untreated rice.

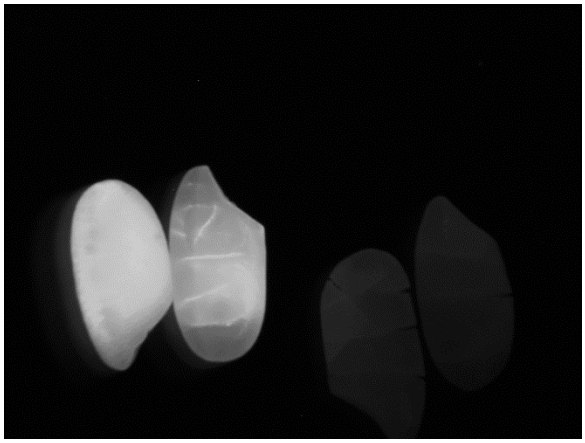

**Figure 4: RFP-SBP coated rice under Fluorescent Microscope:** The rice on the left was incubated for five hours in a solution containing the Red Fluorescent-Starch Binding fusion protein and the rice on the right was simply treated with water. Both rice samples were washed after their treatment. The RFP reaches excitation at 584 nm and emission is at 607nm.

## Positive Control

Two samples of rice were incubated in pink protein solutions for 4 hours each. One sample was treated with red fluorescent protein only while the other sample was treated with the red fluorescent-starch binding fusion protein. Samples were examined after both two and four hours of incubation under a fluorescent microscope set to make RFP fluoresce. All samples fluoresced brightly, with the RFP-SBP sample only fluorescing slightly brighter than the RFP only sample.

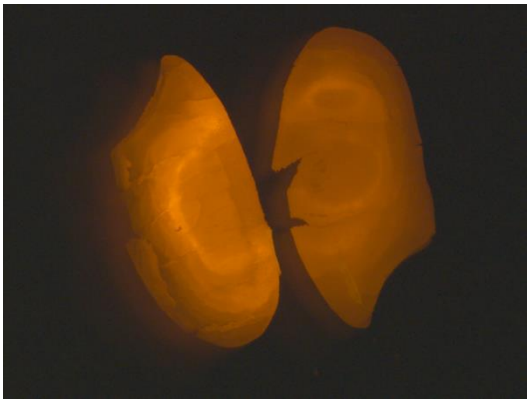

**Figure 5: RFP and RFP-SBP coated rice under Fluorescent Microscope:** Rice treated for two hours in RFP and RFP-SBP examined under a fluorescent microscope (excitation 584 nm, emission 607nm). Rice of the left is treated with RFP-SBP. Rice on the right is treated with RFP only. Both samples were washed before and after protein treatment.

## Protein Spray

Crude red fluorescent protein and crude red fluorescent-starch binding fusion protein solutions diluted to have equal fluorescence are sprayed onto dry rice, allowed to dry, and washed before being placed under a fluorescent microscope set to make RFP fluoresce. The rice treated with fusion protein glows the brightest, the RFP treated rice glows, and the water treated is nearly invisible. To the naked eye, the fusion protein treated rice is slightly pink while the other two samples appear simply white.

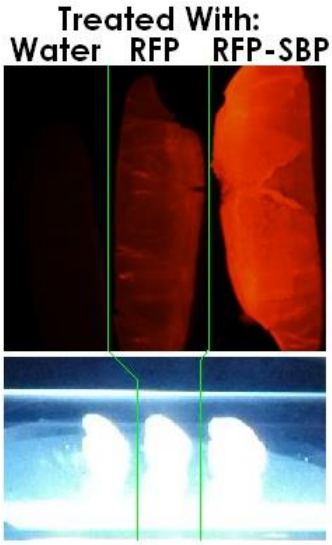


**Figure 6: Water, RFP, and RFP-SBP sprayed rice under Fluorescent Microscope:** The top photo was taken under a fluorescent microscope (excitation 584 nm, emission 607nm). The lower picture shows the samples under white light. An atomizer was used to spray rice with the following treatments: water, crude *E. coli* extract containing RFP, and crude *E. coli* extract containing RFP-SBP. After treatment the rice was air dried and washed before being viewed under the microscope.

# Discussion

## Fusion

The mRFP1-cbm21 construct was confirmed to be present in colonies 3, 4, 6, 7, and 8. Each of the colonies appeared red under normal light indicating the presence of a functional mRFP1 gene within them. The subsequent PCR utilizing the primers SBPsense and SBPanti was done with the purpose of confirming the presence of the cmb21 gene within the colonies. The size of cbm21 is 361 base pairs in size. As Figure 3 shows, the colonies specified above contain bands between 300 and 400 base pairs strongly suggesting the presence of the gene. This information all together suggests the successful fusion of mRFP1 and cbm21 as well as their presence within the colonies.

## Protein Harvest and Usage

As shown in Figure 4, the fusion protein appears to bind to rice and remain on the rice through washing. This does not necessarily relate to the functionality of the starch binding domain. Wet rice is sticky due to the hydroscopic nature of starch. This shows that binding occurs, but not that it is due to the starch binding domain.

## Positive Control

A positive control, Red Fluorescent protein, is applied to rice samples as done previously to test how proteins without starch binding domains interact with rice. Samples of RFP treated rice and RFP-SBP treated rice are compared at two hours and four hours. Samples at four hours appear identical, while, as Figure 5 shows, samples incubated for two hours appear slight different. The RFP-SBP treated rice appears to have more protein attached to it at this point. This difference at two hours when compared to at four hours suggests that the starch binding domain is more efficient at binding than protein without starch binding domains. Red fluorescent protein

is binding to the rice in a comparable fashion to RFP-SBP. This is likely due again to the hydroscopic nature of starch.

## Protein Spray

A new approach is taken with these samples to reduce water interactions the rice and protein. A sprayer is used to apply water, RFP, and RFP-SBP crude protein extracts to dry rice. Water acts as a negative control showing how normal sprayed rice appears under the fluorescent scope. Red fluorescent protein acts as a positive control, depicting how proteins with no starch binding domain interact with the rice. The rice is dried, washed, and dried again before pictures are taken under the fluorescent microscope. As Figure 6 shows, a clear difference is seen between the binding of protein with and without the starch binding domain. The RFP-SBP treated rice appears significantly brighter than the RFP treated rice indicating a larger concentration of protein and the effectiveness of the starch binding domain. This test also suggests the ability of proteins to bind to rice and remain bound without a starch binding domain though the relative binding strength would likely be very variable as the amino acid sequence of each protein is unique.

# Conclusion

This model system demonstrates the viability of cbm21 fusion proteins as tools in rice fortification. The starch binding domain binds well to rice and remains bound through washing. The next step is to create constructs with nutrient binding domains, such as thiamine binding protein, or essential amino acid rich proteins in addition to the starch binding domain. Interestingly, it may even be an option to investigate the ability of nutrient binding proteins and amino acid rich proteins to bind to rice without the aid of a starch binding domain. Though, the use of a starch binding fusion protein is more reliable and provides stronger binding to rice than protein lacking a starch binding domain.

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