5-2018

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05/18

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Honors Thesis

State University of New York Environmental Science and Forestry

May 2018

Abstract:

The use of injectable vaccines and pharmaceuticals pose logistical problems in many parts of the world. This problem has previously been investigated using various plants to produce pharmaceuticals, which would eliminate the problem of injection sterility. However, concentration determination in the harvested plants still pose a critical problem. For other medicines that might be cytotoxic, there is the additional problem that production in the living plant tissues could kill the plant before they could produce enough compound to be economically viable. The solution proposed by this project focused on the use of the promotor for a 58 kD protein in *Capsicum* to target pharmaceutical production to the ripened fruit of the red pepper plant. Due to time restraints in the lab this experiment and paper will focus on the isolation which can then be used in the future to begin work on the promotors. This avoids the issue of cytotoxicity as the promotor drives protein synthesis only in the final ripening states of the fruit, which can be harvested when ripened, thus preventing the entire plant from dying. These peppers can be harvested, ground and dried, providing a sample that can be tested for concentration and uniformly allocated into capsules. The protein has thus far been isolated from the chromoplast of the *Capsicum* fruit using a sucrose gradient centrifugation using a modified method for plant isolations. The next step will be the isolation of both mRNA and genomic DNA to search for the promoter regions using a cDNA clone for the protein of interest. Once the promoter regions are identified a DNA cassette can be produced containing a marker gene and inserted into a range of fruit bearing plants to determine which fruits produce the highest level of the marker, and also the timing of the marker. The final goal would be the insertion of a pharmaceutically relevant gene into the DNA cassette, and create transgenic plants that produce the desired compound only at the time of ripening thus preventing the entire plant being killed

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Introduction:

Vaccines pose both a social and logistical issues in today's world. The social impacts following the 1998 Wakefield study spurred the most recent of a long line of anti-vaccination movements. Driven by distrust of government and pharmaceutical companies, denialism and cultural beliefs (Chapman, 2010) diseases are spreading that could be easily preventable. The effect of the internet, specifically social media has not counteracted these debilitating effects. Fear of vaccines containing poisons or toxins, false correlations between vaccines and autism or the disease itself, and promotion of home or natural remedies that claim to be more effective spread like wildfire on the internet and the social media platforms it provides, increasing the range of the statements while providing little to no check to the truthfulness of these claims (Kata, 2009). This has led to an outbreak of five preventable diseases that had previously been contained, all spurned from the fear of current vaccination and injection methods. The logistical issues provide a more direct impact on those willing to receive the vaccine. A common worldwide problem is the sterility of the needle and ensuring that each injection is doing no harm to the patient. In countries and areas where materials are scarce it may be near impossible to have one needle per patient. A more pronounced problem in developing countries is the current method of transporting vaccines any distance is the Cold Chain. Vaccine temperature must be kept with 2 and 8 degrees Celsius, otherwise the vaccine can be rendered impotent or even cause an adverse effect in the recipient (Fernando, 2013). The storage devices used can hold this optimal temperature, but only when used properly. A study done by Thakker and Woods showed that 75% of the studied refrigerator units exposed their vaccines to temperatures above 16°C to subzero, well beyond the range of suitable temperatures. This is due to both malfunctions with the equipment and a lack of training for the staff (Thakker, Woods, 1992). While training and

technology has improved, these issues persist albeit less often than when this study was conducted. Due to this, vaccine usage in developing countries where these vaccines cannot be produced is limited by the effectiveness of the refrigeration units.

A solution to both problems has been proposed by Doctor Henry Daniell. Plants, in his study spinach, were engineered to produce pharmaceuticals, antibodies and edible vaccines. By engineering the chloroplast of plant, the system ensured high levels of concentration throughout the plant which could then be harvested, dried, ground and packaged into capsules. Issues have arisen from this practice though. Focusing the pharmaceuticals into the chloroplasts of the plant does ensure high levels in the plant, however if the selected pharmaceutical is cytotoxic it could kill the plant, wasting the compound if it wasn't harvested in time. At the very least the cytotoxicity will prevent the plant from propagating and producing further compounds making the process economically inefficient and time consuming (Daniell *et al.*, 2001). Alongside the cytotoxicity, there is an issue with the concentration. The plants engineered by Daniell were producing less than 0.001% of the desired soluble protein that it was engineered for due to under expression of the targeting mechanism, the chloroplast. While chloroplasts are found throughout the plant they are not abundant enough to produce high enough levels of the desired product.

The goal of this research project is to investigate the use of the 58kD protein promotor to control and maximize the production of pharmaceutical products via creation of DNA cassette. The 58kD protein is found specifically in the fruit of *Capsicum* (bell pepper) fruit and only in the final ripening stage. The targeting mechanism of this promotor makes it ideal to target. By using the promotor region of this protein, we can target any pharmaceutical production to the fruit and only at time of ripening. By utilizing this targeting system, we can avoid the cytotoxicity issue, harvesting the fruit at ripening to collect the fruit and preventing the entire plant from dying from

the compound. The expression only in the fruit allows the plant to continue to produce fruit without the need to continually engineer new plants. Since it is found in the nucleus but the protein is targeted to the chromoplast chromoplasts it will also increase the concentration of the product in the plants as opposed to chloroplast genome targeting. Using the fruit of these plants to provide vaccinations in the form of capsules we can quiet fears of 'harmful' chemicals in vaccinations as well as alleviate the troubles arising from transport of the current liquid vaccinations.

Methods:

The goal of this project specifically was the isolation of the protein and perfection of the procedure to do so. A procedure was given to the lab by Dr. Lee Newman who had done research on the isolation of this protein in the past. The initial methodology proposed in the lab was the use of a GR gradient mix to separate the protein from a slurry of blended red bell pepper and GR mix. Using a swinging bucket rotor, the chromoplast would be separated by density from other cellular organelles and could be extracted via pipette and checked for purity with an SDS-PAGE gel.

However, upon analysis of the labs resources it was discovered that there were many materials the lab was missing to follow this methodology. Additional papers were found and analyzed for possible adaptations of the previous procedure. The optimized procedure was derived from a procedure used by Miflin and Beevers (Miflin, Beevers, 1974), which proposed a sucrose gradient alternative to the GR gradient. With the gap in materials filled the procedure

used by Dr. Newman was could be altered and followed to produce the results presented later in this paper.

Sucrose gradients were prepared with 4mL each of 1.45M, 0.84M and 0.45M solutions layered manually on top of each other in a 15mL centrifuge tube. Red peppers were weighed, diced and placed in a bender along with 50-100mL of 1x GR mix. The peppers were blended in 3-5 short bursts. The slurry was filtered through three layers of Miracloth into a beaker. The filtrate was divided into 50mL centrifuge tubes and centrifuged at 1480 rpm in a refrigerated fixed angle rotor for 10 minutes. The supernatant was set aside, and the pellets combined into one tube. The pellet was layered on top of two sucrose gradients. The tubes were centrifuged at 3740 rpm for 30 minutes in a swinging bucket rotor. The darkest lower band was collected with a pipette; this band contained the intact chromoplastids. The chromoplasts were suspended in 7 volumes of wash buffer and centrifuged again at 4°C for 20 minutes at 13,500rpm in a fixed angle rotor. The supernatant was saved as well as the pellet which was suspended in 5 volumes of sample buffer. The pellet and supernatant were added to 10mL of bromephenol blue as a tracking dye and run in an SDS-PAGE gel with 1x SDS-PAGE running buffer at 115 V for 105 minutes.

Results:

Fig. 1 Sucrose gradient gel with isolated chromoplast band

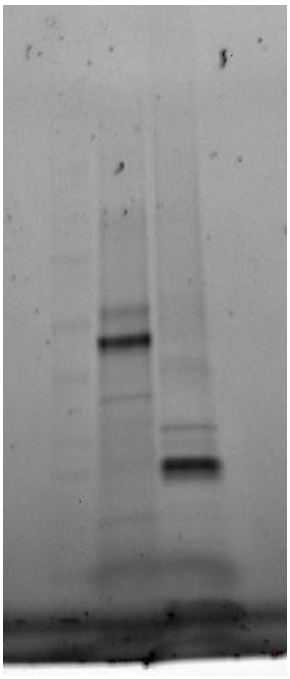

Fig 2. SDS-Page Gel Lane 1 ladders, lane 2 chromoplast, Lane 3 cytosol band (lanes labeled left to right)

Results and Discussion:

The methods from the experiment were originally derived from a previous paper, however due to issues including a lack of materials, in general we were forced to modify the procedure. The major change spurred by this was from the original gradient mixture to a sucrose gradient. This of course also discarded or changed many of the solutions previously prepared, or that the procedure was requiring us to prepare, to fit the new procedure.

Figure one shows the isolation of the bands after centrifugation in the sucrose gradient. The procedure outlined by Miflin and Beever identified where the band of desired chromoplasts would be located within the gradient. The orange band separated from the rest of the color in the gradient was identified to be this location. However, observing the bands it can be seen that the bands were not cleanly separated from the rest of the plant material leaving the possibility of contamination from other plant components. This band was extracted from the rest of the gradient with ease and used in the SDS-PAGE gel, Figure two.

The protein was successfully identified in the red pepper fruit. Seen in the Figure 2, a 58kD protein was extracted from the chromoplasts of the plant and seen in higher amounts than in the solute, the cytosol of the cells. Since the 58kD protein of interest is targeted to the chromoplast once it is translated by the ribosomes we expect to find most of it here with trace amounts in the cytosol (Hadjeb *et al.* 1988). The results of the SDS-PAGE gel provide enough evidence to suggest that the protein that has been extracted is the protein of interest and, after further testing to ensure purity, the experiment can continue into its next stages.

A major problem with this gel was the faded lane showing the bands of the ladder. While they are observable they are faint which leaves room for error in interpretation. While the gel was interpreted for results in the lab more extractions and gels would have to be run to insure the band interpreted as the 58kD protein is indeed the band we believe it to be. The two darkest bands in the cytosol and chromoplast lanes are Rubisco and 58kD protein respectively. A faint band for the 58kD protein can still be seen in the cytosol lane which is expected due to the protein being translated in the cytosol and the likely possibility of not all of the protein would be found in the chromoplast. This also explains the lack of a visible band corresponding to chloroplast proteins in the chromoplast lane.

To optimize the procedure used for chromoplast extraction, more time in the centrifuge to create cleaner bands in the gradient would aid in isolation. The methods could also be improved on by slight alterations to the samples loaded onto the lanes of the SDS-PAGE gel. More samples would allow for a more in depth and accurate analysis of the results and by darkening the ladder to ensure accurate interpretations of the experimental lanes.

Now that the protein has been successfully identified the next step is the isolation of the promoter region for the protein. The first step of isolating the promotor sequence is development of antibodies for the 58kD protein. These antibodies can then be used to probe for the 58kD protein within a cDNA expression library. cDNA can then be used to locate the genomic DNA region. Once found, the DNA for the protein can be screened for upstream regulatory regions, the promotors, which can be isolated for use in creation of the DNA cassette. Once the DNA cassette has been produced it will be inserted into red pepper embryos which will be grown, and fruit tested for the expression of a marker gene. The gene regulatory regions can then be linked to genes encoding vaccine-related proteins. Plants will be transformed and grown, and the fruit

tested to ensure the desired pharmaceutical compound has been produced. Those with the highest production will be bred to maximize the production of the product.

While this experiment is still in its preliminary stages it possibilities for the medical field are endless. The creation of the DNA cassette would allow the insertion of any genetically derived pharmaceutical product. Any protein, nucleic acid, carbohydrate, or fatty acid that a plant has the means to produce can be produced and collected for distribution (Daniell *et al.* 2001).

Conclusion:

With the protein successfully identifie,d the production of the targeted DNA cassette can truly begin. With the previous work done in the lab of Henry Daniell it has been shown that plants can reasonably be expected to produce the products we have need of, albeit at a lower concentration than what is needed and with chance of plant death. Whether or not the promotors and targeting sequences can accurately target the pharmaceutical remains to be seen and the progress of this experiment will not reach that point for a few years. However, the benefits offered by this project, protection against cytotoxic products and increased concentration of the product within the plant, could improve on the project of Henry Daniell. Introduction of the pharmaceutical into the plant's internal metabolism allow for easy collection of the fruit, which can then be dried and ground. Once tested for concentration the capsule pills can be packaged and labeled to be shipped around the world for easy use. This could potentially solve the Cold Chain problem for vaccines once and for all.

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